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REVIEW

Respiratory Syncytial Virus: Infection, Detection, and New Options for Prevention and Treatment

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SUMMARY Respiratory syncytial virus (RSV) infection is a significant cause of hospitalization of children in North America and one of the leading causes of death of infants less than 1 year of age worldwide, second only to malaria. Despite its global impact on human health, there are relatively few therapeutic options available to prevent or treat RSV infection. Paradoxically, there is a very large volume of information that is constantly being refined on RSV replication, the mechanisms of RSV-induced pathology, and community transmission. Compounding the burden of acute RSV infections is the exacerbation of preexisting chronic airway diseases and the chronic sequelae of RSV infection. A mechanistic link is even starting to emerge between asthma and those who suffer severe RSV infection early in childhood. In this article, we discuss developments in the understanding of RSV replication, pathogenesis, diagnostics, and therapeutics. We attempt to reconcile the large body of information on RSV and why after many clinical trials there is still no efficacious RSV vaccine and few therapeutics.

KEYWORDS diagnostics, epidemiology, experimental therapeutics, immunization, respiratory syncytial virus, viral pathogenesis

INTRODUCTION

Respiratory syncytial virus (RSV) was discovered more than 50 years ago (1, 2), and it has since been identified as the most common cause of acute respiratory tract infections in infants (3, 4). Even with an increased understanding of the burden of RSV in the elderly (5–9), there is still a relative lack of knowledge on RSV infection and transmission in this group compared to pediatric patients. Consistent with the massive disease burden that is posed by RSV infection, there is a large body of information that has been elucidated on RSV replication, pathogenesis, and transmission. Despite all that we know about RSV virology, there is a relative paucity of strategies available to prevent and treat RSV infection. We propose that the limited therapeutics and RSV disease burden have been due to several obstacles identified in this review: RSV detection and surveillance are not standardized on a global level, RSV detection approaches historically have not been widely accessible, prophylaxis strategies are inefficient, and there have been few successful RSV antiviral and RSV vaccine strategies brought to market.

This review will identify improvements in the understanding of RSV pathophysiology, diagnosis, and surveillance as well as some of the obstacles to experimental development of RSV therapeutics and vaccines. Importantly, vaccine development was

first hampered by disease exacerbations that occurred when RSV vaccines were prepared by formalin inactivation (10–12), resulting in enhanced disease (12). Since the first RSV vaccine trial, several vaccine preparations have undergone clinical trials, but none has been brought to market. For reasons that we discuss in this review, new approaches in vaccine design and efficacy testing will have to be developed and tried in order to develop a truly efficacious vaccine. Even if an effective vaccine is realized and made available for mass distribution, its implementation must go hand-in-hand with the use of more accessible and specific assays to monitor vaccine efficacy in clinical settings. Understanding of the replication cycle of RSV has led to development and now the clinical testing of new therapeutics to treat those most susceptible to RSV infection. We thus believe that strengthening the linkages between basic and clinical virology will improve our ability to control RSV infections in the future. In this review, we try to reconcile the multitude of different aspects of RSV biology and highlight the principal hurdles in preventing and treating RSV infection.

THE VIRUS, PRONE TO GENETIC CHANGE

Human respiratory syncytial virus types A and B of the species *Human respiratory syncytial virus* are found within the genus *Orthopneumovirus*, family *Pneumoviridae*, order *Mononegavirales*. Structurally, human respiratory syncytial virus is an enveloped, spherical virus with a diameter of approximately 150 nm. In addition, filamentous species capable of reaching several micrometers in length have also been observed. Like the spherical forms, the filamentous virions are infectious (13). The RNA genome is packaged into the viral particle as a nonsegmented negative-sense molecule and codes for key internal structural proteins (matrix protein [M] and nucleoprotein [N]), proteins required for a functional polymerase complex (phosphoprotein [P] and polymerase [L]), nonstructural proteins involved in evasion of the innate immune response (NS-1 and NS-2), externally exposed transmembrane glycoproteins (small hydrophobic protein [SH], glycoprotein [G], and fusion protein [F]), and the regulatory M2 proteins (M2-1 antitermination protein and M2-2, involved in transcription/replication regulation) (14). The RNA-dependent replication cycle of RSV is significant because it is error prone and there is no proofreading mechanism. This allows for the rapid generation of single nucleotide polymorphisms (SNPs) and other mutations which allow for changes in virus virulence and avoidance of potential future antiviral agents or vaccines (15).

Overall Impact: Mortality and Health Care Costs

RSV causes significant pediatric and adult morbidity and mortality, which have a significant economic impact on health care systems. A meta-analysis recently reported that in 2005, between 66,000 and 160,000 children less than 5 years of age died of RSV infection or of complications directly related to RSV infection (16). The majority of these deaths occurred in developing countries (16). Underreporting in meta-analysis due to conservative study exclusion criteria and analysis and the fact that most deaths occur in developing countries likely means that the actual mortality rate due to RSV infection is higher than what was reported (16). However, in the United States, deaths due to RSV infection are relatively low; in those aged less than 2 years, the mortality rate was between 3 and 4 per 10,000 hospital admissions (17). The majority of these deaths were associated with prolonged hospital stays and at least one comorbidity (17). In the United States, it is estimated that 11,000 to 17,000 adults die from RSV infection annually, with approximately 10 times that number of patients hospitalized in 1 year (9, 18). RSV infections in adults are usually not primary infections and are predominantly mild to moderate in severity unless patients have an underlying risk factor such as being immunocompromised, having an underlying chronic pulmonary or circulatory disease, living in a long-term care facility, or being frail (18, 19).

The mortality rate of RSV in transplant recipients is significantly higher than in the general population. Some recent studies have identified a 30 to 100% mortality rate due to RSV infection in solid organ and bone marrow transplant recipients, particularly when infection has occurred within a few days after transplant surgery (20–22). In lung

transplant patients, lower respiratory tract infections (LRTIs) with RSV can be a risk factor for bronchiolitis obliterans (23, 24) and death in up to 20% of cases (20). RSV infection in allogeneic stem cell transplant patients leads to LRTI that is associated with an increased risk of death in up to 70% of those infected (21, 25). In pediatric transplant patients, not all infections are disastrous and outcomes vary broadly from uneventful resolution to hospital admission, with a smaller subset admitted to the intensive care unit (ICU) (22).

The health care costs resulting from the hospitalization of RSV-infected patients are significant. A study that investigated the costs associated with RSV infection in pediatric patients in Canada estimated the yearly cost of RSV to the health care system at 18 million Canadian dollars (1997) (26). In the United States, these costs are much higher at over 600 million dollars per year in total health care due to RSV infections in pediatric patients and the elderly (1999 to 2004) (9, 27). Of course, these cost estimates do not take into account the lost productivity and wages of parents who must stay at home to care for a sick infant or child. In summary, although the mortality rate due to RSV infection is low in developed countries, the societal burden of caring for the ill and health care costs due to RSV infection are very high.

WHO IS AT RISK?

The majority of those who are hospitalized with RSV are infants and toddlers (3). However, adults with comorbidities and the elderly are also at increased risk from RSV infection (5, 28).

Infection in Infants

It is estimated that over 95% of children have been infected with RSV by 2 years of age (4). A recent study on patients under the age of 5 years suggested that RSV infection, among a broad array of pathogens, is the single greatest causative factor of radiographically diagnosed pneumonia in the United States (29). Those less than 2 years of age were particularly at risk. The only reported independent risk factors for hospitalization from RSV infection are prematurity and young age, and therefore, "control strategies targeting only high-risk children" will have very little effect on controlling the burden of RSV infection (4).

One of the primary reasons that the very young are at greatest risk from RSV infection is the high surface area-to-volume ratio of developing airways. Humans are born with almost all of their airways and alveoli (30), and so, the lumen of the bronchioles in the airway is relatively smaller than that of an adult and thus more prone to obstruction (31). Complicating lower respiratory tract virus infections is the smooth muscle hyperreactivity that coincides with inflammation of the airway and further constricts already small airways (32, 33). Therefore, inflammation of developing airways makes them more susceptible to increased airway resistance that presents as wheeze and croup (31).

Severe acute viral lower respiratory tract infection (LRTI) can lead to chronic sequelae. This is predominantly in the form of asthma, but viral LRTI has even been proposed as an etiology of chronic bronchitis (34), obstructive pulmonary disease (35–37), and idiopathic pulmonary fibrosis (38, 39). In a retrospective study of preterm infants with viral LRTIs, significantly elevated airway resistance remained after 1 year of follow-up (40). RSV infection was the most prevalent lower respiratory tract infection that was detected among a panel of common respiratory viruses (40). The increased resistance is most likely a product of the airway inflammation and an inherited hyperreactive profile of the infants due to viral infection. As we will discuss below in this review, there is a hypothesized mechanistic link between RSV LRTI as an infant and the development of asthma later in life. Most notable is the recent discovery of the RSV-induced interleukin 33 (IL-33)–IL-13 axis that may potentiate the development of long-term hyperreactivity in the airway (41, 42), which we discuss below in more detail in "A Causal Link between RSV Infection and Asthma." In summary, there have been recent significant developments in our understanding of the mechanistic queues that

are triggered by viral infection that can lead to chronic sequelae such as asthma and other obstructive pulmonary diseases.

Epidemiological Considerations

While the overall single greatest risk factor for severe RSV infection is age (4), several epidemiological studies have identified factors that can compound the severity of RSV infection. The most notable contributing factors are overcrowding, exposure to smoke (cooking and tobacco), evidence of asthma in the mother, and lower socioeconomic status (43–46). In developed countries, previously healthy children also experience severe lower respiratory tract infections, with those infants suffering comorbidities particularly at risk (3, 4).

Comorbidities

Although age is the single biggest susceptibility factor during RSV infection, there are notable factors that significantly contribute to the risk of RSV infection. In no particular order, these include prematurity at birth, low birthweight (<2,500 g) (47), underlying immunologic disorders either natural or acquired, genetic and chromosomal abnormalities (e.g., Down syndrome), the presence of other pulmonary disease processes, neoplasia, and defects of the heart and/or lung structures (48, 49). At particular risk are children with congenital heart defects (reviewed in reference 50). In those heart abnormality patients infected with RSV, the mortality rate was as high as 37% in the 1970s. Treatment with ribavirin (51), improved pediatric intensive care, and surgical management of congenital heart disease have since decreased the death rate due to RSV infection in these patients to less than 3% (50). Risk factors for death due to RSV in pediatric intensive care units include Down syndrome and evidence of nosocomial infections (52). Given the importance of the immune response to clearing RSV infection, immunocompromised children are at an elevated risk from infection. In summary, disabled children are at an elevated risk during RSV infection and preventative measures should therefore be taken in their respective care units.

Increased Susceptibility to RSV Infection Due to Pharmacological Immunosuppression

Those who are immunosuppressed are at enhanced risk of severe RSV infections (4). This is because neutralizing antibodies afforded by humoral immunity mediate control of RSV infection and clearance of infection is correlated with a robust Th1 cell-mediated immune response (53–56). For example, immunosuppressive antirejection drugs that are commonly prescribed to transplant recipients, like tacrolimus, sirolimus, and cyclosporins, suppress the Th1 arm of the immune response by preventing the activation of T cells. In an immunocompromised state, these patients tend to suffer from a myriad of different opportunistic infections that can be parasitic (57), fungal (58), bacterial (58), and viral (59) in origin. More recently, it has come to light that sirolimus, but not tacrolimus, inhibits B cell differentiation into antibody-producing plasma cells (60, 61). Plasma cells produce the neutralizing antibodies against RSV that are critical for controlling and helping to clear virus infection. Though no particular pathogen was detected specifically, postoperative sirolimus use in transplant patients was predictive of lung infection in heart transplant patients (62). Antirejection drugs that prevent development of graft-versus-host disease in transplant recipients also inhibit the same B cells and Th1 arm of the immune response, which are necessary to clear RSV infection. Thus, any deficiency in either of the humoral or cell-mediated arms of the immune response will lead to more severe viral infection and pathology than those in the immunocompetent.

The Elderly: Susceptibility to RSV

RSV is a significant cause of influenza-like illness (ILI) in those 65 years of age and older worldwide (63, 64; reviewed in reference 5), which has been recognized as a significant cause of morbidity and mortality in the elderly since the 1980s (7, 8). In one

of the most recent studies, RSV was the third greatest cause of ILI in the elderly, next to enterovirus and influenza virus infections (63). However, RSV was the second greatest cause of hospitalization, as elderly patients who were positive for RSV infection were twice as likely to be hospitalized as the patients who were positive for influenza A virus (63). In summary, it is clear that RSV is a prevalent and very serious respiratory pathogen in those 65 years of age and older.

What is less clear are the factors that make the elderly more susceptible to RSV than the rest of the adult population. Declining immune systems and lower RSV-specific serum immunoglobulin (Ig) and nasal IgA titers are associated with susceptibility to RSV in the elderly (65, 66). However, it is unclear whether the individuals reported were more susceptible to RSV infection throughout life or whether humoral immunity decreased as a result of age-related senescence. In other words, are a subset of adults who have been more susceptible to RSV throughout life being hospitalized due to RSV infection and ailing health, or does one become more susceptible to RSV infection with age?

In the elderly, gamma interferon (IFN-γ) expression, as a marker of Th1 immunity, is increased relative to the general population, but there is a decrease in the amount of IL-2 that is produced (67–69). Even though markers of Th1 immunity are elevated, a loss of IL-2 renders one less capable of expanding T cell subsets. Reduced levels of a mitogen, like IL-2, will blunt the ability of RSV-specific T cells to expand during an RSV infection. Therefore, the elderly may be more susceptible to RSV infections because of a blunted immune response as in transplant recipients, although through different mechanisms. The immune response to RSV infection is covered in additional detail in "Immune Response to RSV Infection."

In summary, those with suppressed immune systems, particularly surrounding the function of humoral and Th1 cell-mediated immunity, are at significantly elevated risk of morbidity and mortality related to RSV infection.

RSV REPLICATION

There has been significant progress in the understanding of RSV entry, replication, and egress during the past decade alone. A comprehensive understanding of host-pathogen interactions and viral replication provides the basis of robust diagnostics and the development of antiviral therapeutics. In the section below, we highlight the studies that we think provided the most significant progress in our understanding of RSV replication.

Entry of RSV and Its Host Cell Receptors

Obvious targets for therapeutic development are the receptors that viruses bind to trigger entry into the host cell. There have been many candidate cellular receptors described for RSV entry, including annexin II (70), CX3 chemokine receptor 1 (CX3CR1) (71, 72), epidermal growth factor (EGF) receptor (73), calcium-dependent lectins (70), Toll-like receptor 4 (TLR4) (74, 75), intercellular adhesion molecule 1 (ICAM-1) (76), nucleolin (77, 78), and heparan sulfate proteoglycans (HSPGs) (79). Some receptors like EGF are purportedly used by only certain strains of RSV (73). It is also interesting that of these receptors, annexin II, HSPGs, and C-type lectins (including surfactant proteins A and D, which are soluble and bind to RSV prior to cellular attachment) have been implicated in binding the carbohydrate-rich regions of the RSV-F and -G proteins. This is of particular importance to consider prior to experimentation because culturing RSV in different cell lines (Vero versus HEp-2) can alter the glycosylation patterns of RSV-F and -G, which in turn alters the infectivity both *in vitro* and *in vivo* (80–82).

The RSV-G glycoprotein binds HSPGs (79), which are abundant on numerous cell types and especially so on many immortalized cell lines, including HEp-2 cells. However, HSPGs are not detectable on the apical surface of well-differentiated ciliated epithelial cells (83), the primary site of RSV replication in patients (84). This implies that the proposed interactions between RSV-G and HSPGs may be an *in vitro* artifact that does not translate *in vivo*. RSV-G also binds to the CX3C chemokine receptor 1 (CX3CR1)

expressed on the apical surface of ciliated bronchial epithelial cells (71, 85, 86). Although the RSV-G glycoprotein is dispensable for infection (84), many initial studies characterizing the protein were done using cell lines that express high levels of HSPGs (which also bind RSV-F). Recent studies using differentiated human airway epithelial cells have described a more essential role for RSV-G (71, 85, 86). When tested *in vivo*, mice deficient for CX3CR1 were significantly less susceptible to RSV infection (71). Additionally, the interaction between RSV-G and CX3CR1 induces cellular signaling because it is capable of mediating leukocyte chemotaxis (71, 85, 86). Taken together, it would appear that the interaction between RSV-G and CX3CR1 is important both for attachment of viral particles to the cell surface and potentially for downstream signaling events resulting from the interaction.

In 2011, we reported on nucleolin as a receptor for RSV that binds to RSV-F (77). Expression of human nucleolin on insect cells that are not normally infectible by RSV made them susceptible to infection (77). Although nucleolin is a predominantly nucleolar protein, a small fraction can be found on the cell surface *in vitro* (87) and *in vivo* (88). This cell surface nucleolin has been implicated as a receptor for a number of viral and bacterial pathogens along with various growth factors (reviewed in reference 89). Since nucleolin has been found on the surface in greater quantities on actively dividing cells, this may play a role in the preferential infection of the lower respiratory tract in young children because alveoli continue to grow until about 2 years of age, which is also when RSV infections decline significantly (4, 90–92). However, nucleolin is a leaderless protein, meaning that it is not expressed on the plasma membrane of the host cell in a typical fashion, nor is it understood how this protein is even tethered to the plasma membrane.

A recent report by Holguera and colleagues supported the interaction between RSV and nucleolin expressed at the cell surface (78). However, the authors reported that pretreating RSV with heparin blocked the RSV interaction with nucleolin (78), which is in contrast to the original report identifying nucleolin as an RSV receptor (77). The discrepancy may be because a different strain of RSV was tested in each of the papers: Holguera et al. used the Long strain (78) and Tayyari et al. used the A2 strain of RSV (77). Alignment (93) of the amino acid sequences of the fusion proteins from these two strains revealed 98.1% sequence identity. One of the few obvious differences is a charged arginine on the Long strain versus a serine on the A2 strain at position 213 of the RSV-F protein. The arginine, given its charged nature at neutral pH, will be more likely to bind to heparin than a serine residue. Amino acid position 213 on RSV-F is membrane distal (94) and thus more likely to interact with a cellular receptor. A comparison of the results from these two papers (77, 78) therefore may suggest that the nucleolin and heparin binding sites on RSV-F are adjacent or overlapping, depending on the strain of virus.

In addition to CX3CR1 and nucleolin, RSV-F and -G glycoproteins bind to a number of other receptors during cellular entry. As shown in Fig. 1, the RSV-F glycoprotein binds to Toll-like receptor 4 (TLR4) that is expressed on ciliated bronchial epithelial cells in the airway (74) and triggers signaling by the virus during entry (75). However, TLR4 on its own is not sufficient for entry of RSV, and so it is not an entry receptor on its own, *per se*. Instead, RSV binding to TLR4 triggers kinase activation that potentiates the entry of RSV particles into target host cells (75), likely by enhancing endocytosis (75, 95). Although TLR4 was not necessary for infection, it was proposed as a mechanism of triggering the cell signaling that is required for RSV entry, and thus, the term signaling receptor was proposed (75). There are a number of other receptors that bind to RSV-F and -G. Thus, the roles of TLR4 as a signaling and tethering receptor for RSV entry are likely carried out by a number of other receptors as well. As with TLR4 binding by RSV, it would be interesting to know whether RSV can induce outside-in cell signaling in epithelial cells by binding to CXC3R1. In other words, are TLR4 and CX3CR1 redundant signaling receptors or are their actions complementary because they are bound by different RSV glycoproteins?

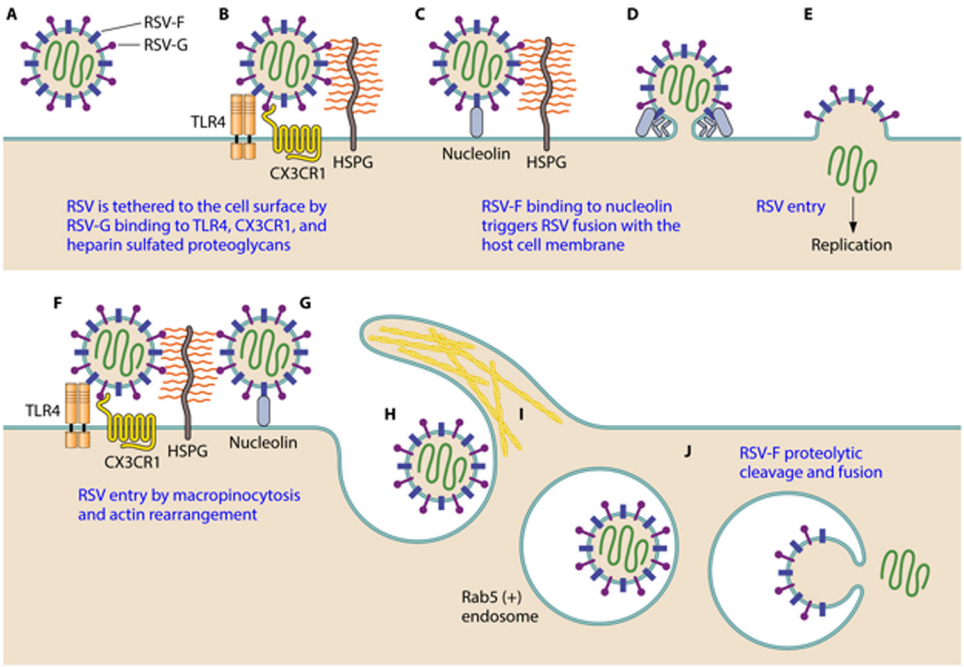

FIG 1 Binding and entry of RSV into the host cell. Candidate receptors of RSV (A) such as TLR4, CX3CR1, and HSPG (B) bind to the RSV-G glycoprotein and act to tether the virus particle to the cell surface. Cell surface nucleolin may also be involved in the entry process (C) by triggering fusion of the virus and host cell membranes by binding to the RSV-F fusion glycoprotein (D). The virion fuses with the cell membrane and enters the cell, one of the last events of virus entry that must take place for successful replication of RSV in the host cell (E). Host cell macropinocytosis of RSV is also a route of entry for RSV (F). It is unclear which receptors are involved in this process (G). Internalization of the virion (H) is dependent on actin rearrangement, phosphatidylinositol 3-kinase activity, and host cell (I) early endosomal $Rab5^{+}$ vesicles where proteolytic cleavage of the RSV-F protein triggers delivery of the capsid contents into the host cells by fusion of the virus and endosomal membranes (J).

During or after receptor binding, the RSV nucleocapsid is internalized by the host cell (Fig. 1). This internalization occurs when the RSV envelope fuses with the host cell at or near the plasma membrane on lipid raft platforms (96), and fusion is triggered in a pH-independent manner. There was a recent report showing that prior to fusion, RSV is internalized in an actin-dependent process called macropinocytosis which also appears to require the host cell protein Rab5 in HeLa cells (95). In the early endosome, furin proteases or cathepsins digest and activate the RSV-F protein, triggering the fusion reaction in the endosome (95). It is not currently known whether nucleolin and CX3CR1 are internalized into Rab5-positive macropinosomes during RSV entry. In fact, it is not known whether interaction with nucleolin is necessary to trigger release of the fusion peptide of the RSV-F protein. There is little known about the trafficking of CX3CR1 in bronchial epithelial cells, but nucleolin traffics through early endosomes in epithelium when it is bound and internalizes ligands.

The wide variety of receptors that RSV has evolved to use for entry into bronchial epithelial cells opens up novel antiviral therapeutic and prophylactic strategies. However, the large number of receptors also indicates that some receptors may share roles with various degrees of redundancy, depending on the strain of RSV tested. As a result, this redundancy and strain-specific variation would hamper the translation of these novel prophylactics and antivirals into broad-use therapeutics. Nevertheless, this obstacle can be minimized by further molecular characterization of any redundancies or variations depending on RSV strain for potential RSV receptors, along with employing effective surveillance strategies to monitor prevailing region-specific RSV strains.

Structure of RSV Fusion Protein before and after Fusion

The RSV-F fusion protein mediates the fusion of the virus membrane with the host cell membrane (Fig. 2). Enveloped viruses require fusion proteins expressed on the

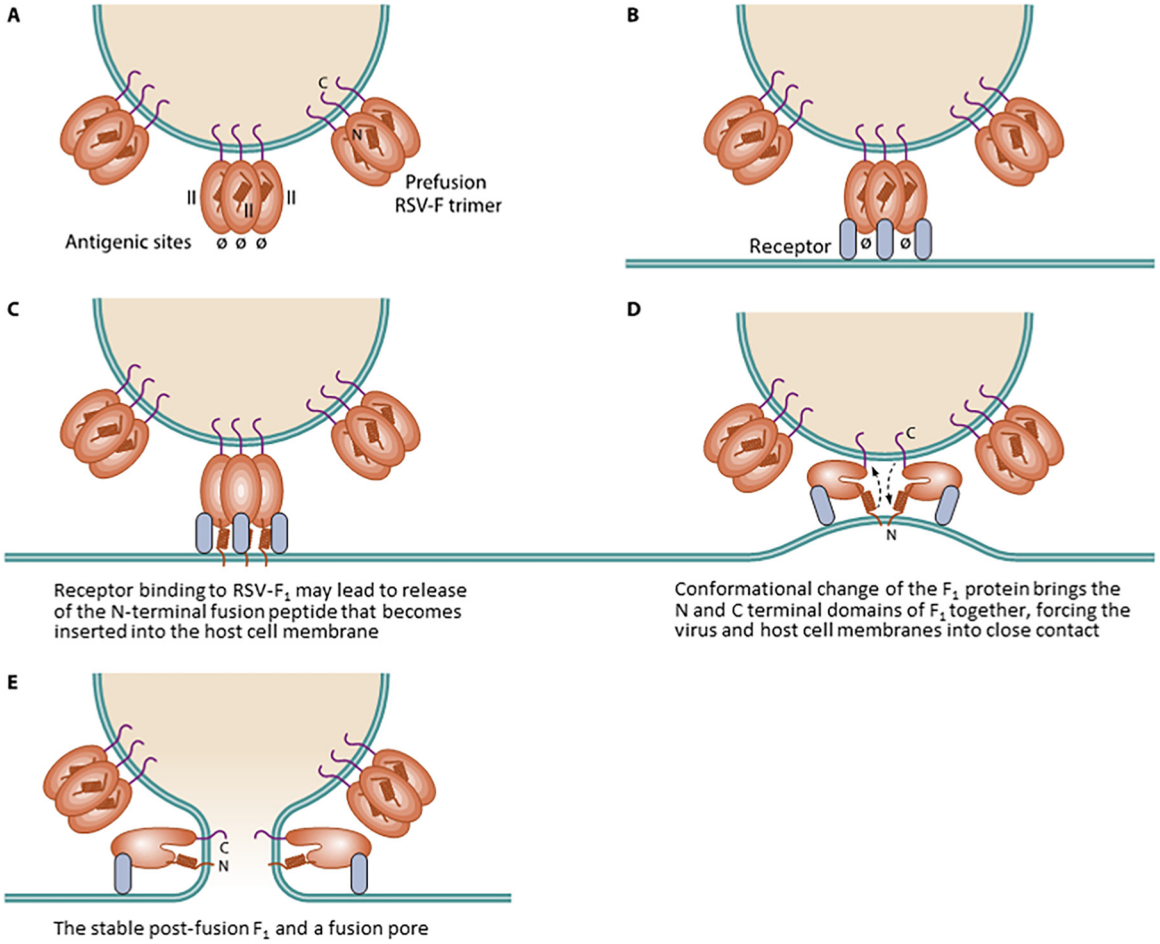

FIG 2 Fusion process between the RSV envelope and cellular membrane. The RSV envelope has multiple protruding RSV-F fusion glycoproteins, anchored via transmembrane domains (A). In the prefusion state, RSV-F exists as a spring-loaded trimer with the major neutralization epitopes shown at the N-terminal region. The major antigenic site Ø exists only on the prefusion trimer and is lost after fusion. Interaction between the RSV-F trimer and a receptor may cause RSV-F to undergo a dramatic conformational shift (B), which leads to insertion of the fusion peptide into the host cell membrane (C) and forcing of the viral and host membranes into close contact (D). Although only two RSV-F monomers are depicted for simplicity, the combined force of multiple RSV-F conformational shifts is required to overcome the thermodynamic barrier of mixing membranes and establish a stable fusion pore for viral nucleocapsid delivery (E).

virion surface to mediate fusion of the virion membrane with the host cell plasma membrane that leads to entry and infection. The fusion protein is needed to overcome the "hydration force" that normally prevents the merging of the opposing virion membrane and the host cell plasma membrane (97, 98). Membrane mixing permits the delivery of the virion capsid into the host cell due to the formation of a fusion pore (Fig. 2). By the very nature of phospholipid bilayer mixing during fusion, the phospholipid constituents of the viral membrane become incorporated into the fabric of the host cell plasma membrane (97, 98).

The structures of the RSV-F fusion protein and its major antigenic sites have been elucidated (94, 99, 100), and they bear a striking resemblance to the core structures of other viral type I transmembrane fusion proteins like influenza virus hemagglutinin. Prior to fusion between the viral and cellular membranes, RSV-F exists as a "spring-loaded" trimer with the major antigenic site Ø exposed (94). Here, we propose a fusion model (Fig. 2) where the RSV-F trimer binds to cell surface nucleolin, which might cause RSV-F to undergo a dramatic conformational shift, during which the major antigenic site Ø is lost (94). Although it has never been reported, a receptor interaction such as binding to nucleolin may lead to insertion of the fusion peptide into the host cell membrane, causing the destabilization necessary to permit membrane mixing. The RSV-F conformational shift additionally forces the viral and host membranes into close

contact. A successful fusion event would require the combined force of multiple RSV-F conformational shifts to overcome the "hydration force" and establish a fusion pore to permit delivery of viral nucleocapsid into the cytoplasm.

Virus Transcription, Translation, and Genome Replication

Elucidation of viral transcriptional processes is essential to develop viral replicon systems that can be used to screen inhibitors and develop antiviral drugs (101). RSV replicons have been used already to develop RSV polymerase inhibitors to the point of clinical trials (102), as we discuss in further detail in "Nucleoside Analogues and Small-Molecule Inhibitors."

Upon completion of entry, the contents of the infecting viral particle are released into the host cell. The viral replication complex then forms on internal cellular membranes (103) and consists of viral and certain host cell proteins (104). The L-polymerase and the P and M2-1 proteins encoded by RSV, along with viral genomic RNA encapsidated by RSV-N protein, are carried in the particle and coalesce to form a replication complex. Host cell proteins are likely contributed by the autologous infected cell but may also be carried in the virus particle from the donor cell from the prior round of replication (105). Proteins involved in virus transcription must be carried in the virus particle to the target cell because RSV is a negative-sense RNA virus and the genomic RNA alone is not infectious, unlike many positive-sense RNA viruses (106). The RSV RNA-dependent RNA polymerase plays a dual role, capable of both transcribing viral mRNA transcripts and synthesizing full-length, positive-sense antigenomes (107).

The RSV genome contains noncoding regions in the 3′ and 5′ termini, called the leader and trailer regions, respectively. Within the 3′ leader sequence, the individual nucleotides important for both replication and transcription have been finely mapped using mutagenesis (107, 108). There is a great deal of similarity in the nucleotides needed for the polymerase to perform either replication or transcription (107, 108); the leader sequence itself can be split into 3 regions going from 3′ to 5′: polymerase initiation site (nucleotides 1 to 15), the RSV-N encapsidation and elongation signal (nucleotides 16 to 34), and transcription signal (nucleotides 36 to 43) (108). This provides support for a model where the polymerase must always initiate at the 3′ end, regardless of whether transcription or replication is taking place (as opposed to internal entry to a gene promoter site during transcription). The 5′ trailer region plays a role in inhibiting the formation of cellular stress granules (SGs) during RSV replication (109). When the trailer region is replicated into positive-sense RNA, the trailer complement is a powerful promoter, similar to the leader region, which drives replication of antigenomes back into negative-sense genomes (110).

During viral transcription, the polymerase complex starts from the 3′ terminus and sequentially transcribes each of the individual viral genes from their own promoters called gene start (GS) sequences, with transcription ending at the gene end (GE) sequence. This occurs in a serial stop-start fashion where the polymerase "scans" the intergenic sequence after a GE signal before initiating transcription on the next GS signal (111, 112). Similarly to host mRNAs, the viral transcripts are capped and polyadenylated before release. The host cell ribosome complex then translates proteins from the viral mRNA transcripts as it would transcribe cellular mRNAs. After an accumulation of viral proteins occurs and through a not completely elucidated mechanism, likely involving the RSV-M2-2 protein (113), the viral polymerase switches from transcribing individual genes to replicating full-length, encapsidated antigenomes where the polymerase is no longer directed by GS and GE signals. Using the trailer region as a promoter, at the 3′ end of the positive-sense antigenome template, full-length negative-sense genomes are replicated for viral assembly and release (110).

A major problem that single-stranded viruses must overcome is being able to initiate replication at the terminus of a nucleic acid strand and maintain an accurate terminal sequence (without an upstream sequence or primer to bind). RSV has evolved an intriguing ability to initiate RNA synthesis in a nontemplated manner during both antigenome and subsequent genomic synthesis. This functions as an innate repair

mechanism, permitting RNA synthesis in a template-independent manner at the correct location (relative to the leader sequence). In the process, 3′ additions are removed (although 3′ additions of 6 nucleotides or greater strongly inhibit replication), and 3′ substitutions or deletions in the first 2 terminal nucleotides are repaired (114–116). This means that the first nucleotide of the nascent RNA molecule begins with an A irrespective of the presence of a U, C, A, or deletion at the 1 position in the template.

The current best-fit model of how the RSV polymerase complex switches from transcription to replication is as follows: despite the ability to correct genomic termini, the RSV polymerase complex does not always initiate at the 3′-most nucleotide (+1 position). The complex is also capable of initiating RNA synthesis 2 nucleotides downstream of the 3′ terminus (+3 position) (117, 118), which is where viral gene transcription is initiated. This +3 initiation often leads to the synthesis of short, abortive transcripts about 25 nucleotides in length. The function (if any) of these short abortive transcripts is unknown, but it is believed that this +3 initiation leads to a subsequent transcription initiation at the first GS signal, which continues transcribing genes sequentially down the genome (118). Combined with the ability of RSV to initiate RNA synthesis in a nontemplate manner, this leads to a model where the polymerase always starts at +3 but full-length replication occurs only if the polymerase is "preloaded" with an A and C (first 2 nucleotides). If the polymerase is not preloaded with an A and C, RNA synthesis instead starts at +3, no RSV-N encapsidation of the nascent transcript occurs, and the complex continues as a transcriptase.

Additionally, the RSV polymerase complex has the ability to loop the terminal antigenomic RNA back on itself in a hairpin and extend the 3′ terminus in a "backpriming" event (117, 119). Although the function of this backpriming is not known, it is speculated to play a role in negatively regulating transcription of antigenomic RNA to genomic RNA, and up to 40% of the antigenomic transcripts detected in infected cells show evidence of 3′ extension (117). The RSV genome is stably present as a helical nucleocapsid encapsidated by a series of RSV-N monomers, each binding 7 nucleotides in a non-sequence-specific manner (120). Furthermore, it has been suggested that interaction of RSV-L with RSV-N can cause a conformational shift in RSV-N that "opens up" encapsidated RNA to be read without physically displacing the RSV-N protein from the strand. However, the presence of this backpriming event, along with the ability to detect RSV genomic RNA in living cells using RNA probes (121), indicates that the RSV genome is not always completely encapsidated, and sections of the genome may be transiently unencapsidated. An ability for RSV-N protein to shift on the RSV strand could explain why, unlike other members of the *Mononegavirales* order, RSV is not bound by the rule of 6: the need for a multiple of 6 nucleotides in the genome in order for the virus to replicate (122). Transient unencapsidation of the RSV genome would also explain why adding more than 5 nucleotides to the 3′ end of the genome strongly inhibits RSV RNA synthesis (114), where the region near the 3′ terminus may be more transiently bound by RSV-N, but adding more nucleotides "buries" this sequence, making it difficult for the polymerase complex to bind.

Role of Inclusion Bodies and Stress Granules in RSV Infection

Large cytoplasmic protein inclusions, termed inclusion bodies (IBs), were first identified in RSV-infected cells using electron microscopy (123). They are distinct features that are observable by histology of lung sections from RSV-infected patients as we discuss in "Gross Pathological Signs of RSV Infection." Small inclusions start forming in RSV-infected cells around the same time as viral protein synthesis begins (about 6 h postinfection), and the IBs increase in size as RSV replication proceeds (124). Although the exact function of IBs during an RSV infection has not been fully elucidated, the inclusions are believed to play a role in viral RNA synthesis and modulating host responses. Within infected cells, IBs are observed in close apposition with cellular membranes, often near the Golgi apparatus (103), and contain all of the components required for viral transcription and replication (RSV-N, -M2-1, -L, and -P and genomic

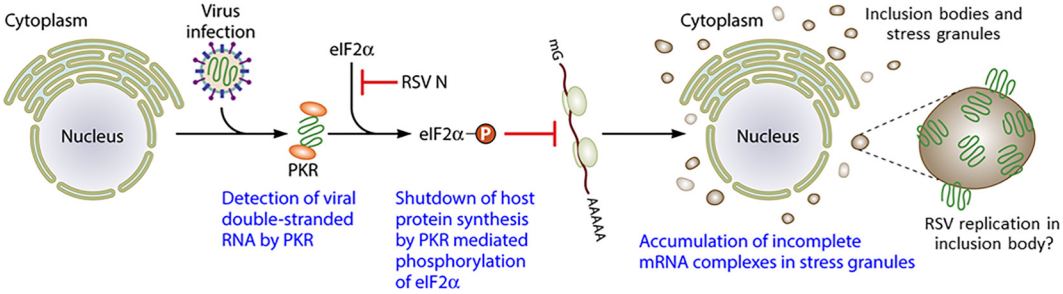

FIG 3 Inclusion bodies and stress granule formation in RSV-infected host cells. Host cell protein kinase R (PKR) detects double-stranded RNA, a by-product of viral replication, and then phosphorylates the translation initiation factor eIF2α. Therefore, stress granule formation is a product of the host innate response against infection that downregulates both host and viral protein synthesis. However, viral countermeasures include preventing the phosphorylation of eIF2α via RSV-N and sequestering stress granule-promoting proteins like O-linked *N*-acetylglucosamine transferase within viral inclusion bodies, thereby preventing stress granule formation. The exact roles of stress granules and their relation to inclusion bodies within an RSV infection *in vivo* are still debatable; in addition, it will be important to determine the impact of RSV-induced cellular stress on the future development of airway diseases such as asthma.

RNA) (125–127). For this reason, IBs are thought to be a primary site for viral RNA synthesis and, as a result, important for the RSV life cycle.

Although expression of RSV-N and RSV-P together is the minimal requirement to form IBs (124, 126), a number of other viral and host proteins have been observed colocalizing with the inclusions during RSV infection. The host proteins that colocalize with IBs include a number of antiviral, chaperone, and signaling proteins such as MAVS (124), MDA5 (124), phosphorylated p38 (128), O-linked *N*-acetylglucosamine transferase (128), and HSP70 (129). The presence of antiviral and signaling proteins within IBs opens up the possibility that these proteins are specifically sequestered within the inclusions to prevent host cell responses that would be unfavorable to RSV infection. This model would help provide an explanation of why RIG-I plays a larger role than MDA5 in the innate immune response to RSV infection (130), since MDA5 is observed almost exclusively in an IB and binds more strongly to RSV-N than does MAVS or RIG-I (124). According to this model, sequestration of O-linked *N*-acetylglucosamine transferase provides a mechanism for RSV to prevent stress granule assembly, since the modification of proteins with O-linked *N*-acetylglucosamine is an important early step in stress granule assembly (131). Sequestering host proteins in IBs to inhibit stress granule assembly is consistent with the negative correlation observed between the presence of large IBs and stress granule formation (128).

Stress granules (SGs) are aggregations of proteins and mRNA that form when the cell is under stress. There has been a particular surge in the publication of studies investigating the interactions of RSV with SGs. In the context of RSV infection, the SGs are produced as a product of the innate immune system response to infection. As shown in Fig. 3, the formation of SGs during RSV infection occurs via protein kinase R (PKR) activation by detection of viral RNA molecules (132). PKR is an intrinsic pattern recognition receptor that detects and is activated by double-stranded RNA (dsRNA), a by-product of RSV replication. When PKR is activated, it phosphorylates and shuts down the activity of eukaryotic initiation factor 2α (eIF2α), which is a necessary component of translation initiation in the cell. When a cell is infected with a virus, PKR is a critical mediator of the innate antiviral response that acts to shut down host and, therefore, virus protein synthesis. However, the RSV-N protein is capable of binding to PKR and preventing it from phosphorylating eIF2α (133). This allows protein synthesis to be maintained during RSV replication.

The RNA molecules that are found in SGs are stalled translation initiation complexes, a product of PKR activation (Fig. 3). The exact function of SGs during viral infection is still debatable. Some viruses have evolved to overcome the SG response, while in other viral infections SGs effectively prevent viral replication (reviewed in references 134 and 135). RSV infection induces SG formation in about 5 to 10% of cells *in vitro* (109, 127,

128), but RSV has evolved mechanisms to overcome the antiviral functions of SGs. For example, the 5′ trailer region of the RSV genome actively inhibits the SG response in infected cells (109). It is possible that the RSV trailer sequence may sequester proteins, such as T-cell-restricted intracellular antigen 1-related protein (TIAR), that are involved in SG formation, which is a mechanism utilized by the related Sendai virus (109, 136). However, RSV with a mutated trailer region still efficiently induces SG formation in TIAR-knockout mouse embryonic fibroblasts (109), which implies that sequestration of TIAR is not the only mechanism that RSV uses to prevent SG formation. Sequestration of O-linked *N*-acetylglucosamine transferase into viral IBs may be another means by which RSV prevents the formation of SGs in infected cells (128). A third mechanism employed by RSV to prevent SG assembly is inhibition of eIF2α phosphorylation by RSV-N binding to PKR (133). There is further evidence that SGs may be coopted by RSV replication machinery, but this is still under debate. The presence of SGs during infection is correlated with small viral IBs compared to a few large IBs in the absence of SGs (128). Transient interactions between RSV genomic RNA and RSV-M2-1 with SGs have been detected, which implies that SGs may play some role in RSV replication (121, 128). Additionally, there has been a report of poor infectivity in cells with a knockdown in G3BP (SG assembly factor) (127). Conflicting reports have shown that cells with a knockdown of protein kinase R could not form SG but had unaltered RSV replication (127, 132). In another report, mutations were introduced to the trailer region of the RSV genome, which resulted in viruses that induced SGs in more than 50% of infected cells and were replication deficient (132). Interestingly, the expression of endoplasmic reticulum (ER) stress markers is increased in asthmatic patients, and addition of a chemical chaperone in the mouse model alleviates asthmatic symptoms (137). The implication of cellular stress for asthmatic symptoms may be part of the mechanism by which RSV infection early in life predisposes individuals to asthma later in life. In summary, though it appears that RSV replication is capable of inducing SG formation, RSV has evolved mechanisms to prevent the formation of SGs. The clinical implications of SGs during RSV infection have yet to be fully elucidated.

MECHANISMS OF RSV PATHOLOGY

Immune Response to RSV Infection

RSV viral load is correlated with the severity of disease, as is the case with many other common respiratory viruses (138, 139). Thus, the severity of infection dictates the degree of inflammation (139). RSV causes a neutrophil-intensive inflammation of the airway during both upper and lower respiratory tract infections in infants (140). Infection can be accompanied by eosinophilia that is particularly marked in the most severe cases of RSV LRTI (11). As we will discuss more in this section, RSV is relatively less cytopathic than other respiratory viruses. It is therefore thought that the majority of damage done to the airway during RSV infection is mediated by the immune response and not by viral replication itself.

Correlates of Immune Protection from RSV

Protection and clearance of RSV infection are likely mediated by a balance of neutralizing antibodies of the humoral immune response and cytotoxic T cells of the cell-mediated immune response (14). Though it is unclear when they arrive at the site of infection, neutrophils contribute the highest proportion of leukocytes in the airways of infants infected with RSV, accounting for 73 to 90% of inflammatory cells (141). In mice, the first cells on the scene of RSV infection in the airways are natural killer cells (142). These are followed by cytotoxic and helper T cells. Cytotoxic T cells kill infected cells and thus help to resolve RSV infection. Consistent with many different types of virus infections, the rise in cytotoxic CD8 T cells is associated with clearance of RSV (143). Deficiencies in CD4 and cytotoxic CD8 T cells have even been associated with fatal influenza virus and RSV infections in infants (144) and the immunocompromised (145). Neutralizing antibody levels rise later in infection and prevent reinfection by opsonizing key viral epitopes required for virus entry and infection. Perhaps of no

surprise is that clearance of virus from the nasal passage has been associated with RSV-neutralizing nasal IgA in infants (146). These studies all argue that both humoral and cytotoxic arms of the immune response are required to control RSV infection, in infants in particular.

Eosinophilic Infiltration of Airways during RSV Disease

Histopathological analysis of autopsy samples demonstrated elevated levels of eosinophils in the airways of infants who succumbed to RSV infection (11), which can be observed in mouse models of RSV infection (147). The primary cause of eosinophil influx during RSV replication is the expression of IL-5 and eotaxin, Th2 cytokines that are induced by the G glycoprotein of RSV. As a result, it has been assumed that eosinophilia was a major contributing factor to enhanced RSV disease, during formalin-inactivated vaccine-enhanced disease in particular (147–151). Eosinophilic infiltration is associated with Th2-mediated allergic asthma and eosinophilic bronchitis (152). However, the roles of eosinophils during RSV pathogenesis have been called into question (153), particularly pertaining to the original autopsy observations that reported variable eosinophil infiltration (11). There was no mention of neutrophils in the pathologist's report (11), which may have been erroneously interpreted as eosinophils being responsible for pathogenesis. Of course, enhanced RSV disease can be observed in the absence of eosinophil infiltration. A recent study demonstrated the same level of RSV disease in eosinophil-deficient mice although there were high levels of IL-5 expression (154). The level of RSV disease was more dependent upon the activation of Th2 subsets than on infiltration by eosinophils. This study therefore suggests that eosinophilia may be a mere indicator of intense Th2 skewing of the immune system to RSV infection and may not have pathological consequences on its own.

Gross Pathological Signs of RSV Infection

Most of the information that is available about the gross pathological signs of RSV infection is observations from bronchoalveolar lavage (BAL) fluid, biopsy, and autopsy samples derived, primarily, from severe cases. There is little published information available on the histopathology of mild RSV bronchiolitis and pneumonia cases. In severe RSV cases, most of the damage to the airway is due to the immune response to RSV infection. Damage is most often concentrated around the bronchi and bronchioles, and the majority of cells that stain positive for RSV are otherwise normal in appearance (155). However, sometimes there are distinct histopathological signs of direct cytopathology caused by viral replication in bronchial epithelial cells, including the presence of intracytoplasmic inclusion bodies and multinucleated syncytial giant cells. As discussed in "Role of Inclusion Bodies and Stress Granules in RSV Infection," granular intracytoplasmic inclusion bodies that stain intensely for RSV antigen have been observed in infected cells of autopsy samples from severe cases (156, 157). Multinucleated syncytial giant cells are a rarer occurrence in most cases (156, 157) and are much more common during *in vitro* culture of RSV. However, multinucleated cells as a result of RSV infection can be quite prevalent in the bronchi and even the alveoli of younger pediatric cases (155), in the immunocompromised (145, 158), and in fatal severe RSV infection cases (157). In some of these severe cases, there is significant involvement of the alveolae, which become clogged by RSV-infected and necrotic debris that has sloughed from earlier-generation bronchi (157) (Fig. 4).

RSV Replicates in Apical Ciliated Bronchial Epithelial Cells

In vitro, RSV does not cause sloughing of cells from air-liquid interface (ALI) culture, unlike influenza virus, which is significantly more cytopathic (159). This is because RSV replicates almost exclusively in apical ciliated cells in stratified ALI epithelium models (reviewed in reference 84). These observations are in contrast to adenovirus and influenza virus, which infect cells at or near the basolateral surface and basement membranes of ALI cultures, causing significant cell sloughing and more severe cytopathic effect (83, 159). *In vitro* studies suggest that RSV is a less cytopathic virus because

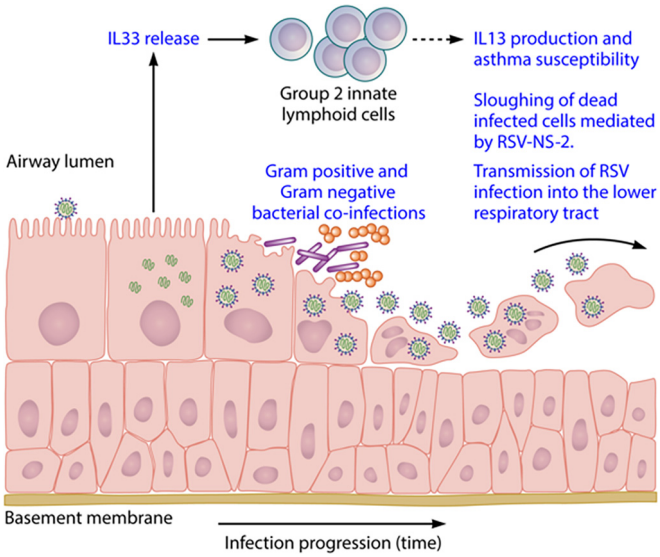

FIG 4 Association of IL-33 with asthma during RSV infection. Based on animal models, IL-33 may be induced in the lungs of infants. IL-33 stimulates group 2 innate lymphoid cells (e.g., nuocytes) to propagate and release the asthma-promoting cytokines IL-4, IL-5, and IL-13. Sloughing of infected ciliated bronchial epithelial cells is mediated by the RSV accessory protein NS-2, which transmits infection into the lower respiratory tract. Bacterial coinfections during RSV infection are common, and prior colonization with potentially pathogenic bacterial species may be a risk factor for severe RSV infection.

it infects only apical cells in the stratified airway epithelium, resulting in superficial damage to the airway lining.

Sloughing of Ciliated Epithelial Cells Caused by RSV Infection

Although RSV does not cause significant cytopathic effect *in vitro*, it causes significant pathology in the airway *in vivo*. In adults, RSV replication continues in the airway for about 8 days (6 days longer than influenza virus) (139, 160–162). In a study that looked at RSV infection in a hamster model, 3 days after infection, there was significant rounding of RSV-infected columnar ciliated cells in the airway (162). These RSV-infected columnar cells eventually sloughed into the lower airway bronchioles, causing obstruction (162) (Fig. 4). The rounding and sloughing effect was mapped to the activity of one of the two RSV accessory proteins, NS-2 (162). Expression of NS-2 from RSV in recombinant parainfluenza virus 3 (PIV3-NS-2) caused an RSV-like sloughing effect in PIV3-NS-2-infected animals (162). These findings are reminiscent of the airway obstruction that occurs in RSV-infected infants during lower respiratory tract infections (144, 162). Perhaps, where this study provides the greatest insight is into the mechanism of transmission of RSV from the upper respiratory tract (URT) to the lower respiratory tract (LRT). Previously, it was not understood how a virus less cytopathic than influenza virus could be transmitted from the upper respiratory tract into the LRT. The study by Liesman et al. (162) therefore suggests a route of infection to the LRT by RSV that is associated with infected ciliated columnar cells that are sloughed into the LRT, mediated by the RSV accessory protein NS-2. The virus associated with sloughed debris can then transmit infection to later-generation bronchi in the LRT (163).

Lower Airway Obstruction during RSV Infection

The cellular inclusions that cause lower airway obstructions consist of mucus, cell debris, and DNA (164, 165). A large proportion of the cellular debris consists of neutrophil infiltration in the LRT during RSV infection (32). In a mouse model, depleting neutrophils during RSV infection resulted in a slight increase in viral load, but there was a significant decrease in airway mucus, which was attributed to decreased tumor necrosis factor alpha (TNF-α) and interleukin-13 (IL-13)$^+$ CD4$^+$ T cells (166). In addition to sloughed epithelial cells and neutrophil-dependent mucus production, neutrophil

extracellular traps (NETs) provide a synergistic cause of the viscous, DNA-rich obstructions. Although traditionally identified as antibacterial in nature, NETs occur when stimulated neutrophils eject their genomic DNA along with antimicrobial proteins like neutrophil elastase and myeloperoxidase (167). These NETs serve to sequester and inactivate pathogens, including RSV (168, 169). Intact RSV particles and purified RSV-F fusion protein induce NETs in a TLR4-, p38 mitogen-activated protein (MAP) kinase-, and extracellular signal-regulated kinase 1 and 2 (ERK1/2) MAP kinase-dependent manner *in vitro* (168, 169). NETs were later observed *in vivo* using bronchoalveolar lavage samples from children with RSV LRT infection and histological sections of lower airway obstructions in calves infected with bovine RSV (168). Despite the ability of NETs to bind viral particles, less than half of the lower airway obstructions observed in infected calves contained viral antigens (168). This opens up the possibility of an immunopathogenic effect that contributes to RSV pathology by exposing host tissues to the histones and myeloperoxidases associated with NETs that are toxic (169). Neutrophils isolated from asthmatic patients responded to RSV viral particles in inoculum by secreting a greater amount of IL-8 (170), and it is known that IL-8 alone can induce the release of NETs (167). Therefore, the production of NETs during RSV infection could also be a mechanism of RSV-induced asthma exacerbations (171).

Exacerbation of Preexisting Airway Diseases

RSV is a major cause of exacerbation of airway diseases like asthma and chronic obstructive pulmonary disease (COPD) (reviewed in references 172 and 173). In contrast, there is not an increase in susceptibility to infection or replication by RSV in adult asthmatics compared to healthy controls (174). However, those at greatest risk of developing asthma, children with wheeze and atopy, experience significantly elevated RSV load compared to healthy children (175). Expression of antiviral immune factors from bronchial epithelium was largely intact as reported in both studies (174, 175). Interestingly, RSV uses entry receptors, TLR4 and CX3CR1 (discussed in the section above), that are upregulated during airway inflammation in both asthma and COPD. Given that bronchial epithelial cells from children with wheeze are more susceptible to RSV infection, it would be interesting to know whether RSV receptors like nucleolin, CX3CR1, and TLR4 are upregulated on these cells. It does appear that the expression of CX3CR1 is mechanistically tied to airway inflammation (176), while TLR4 is upregulated on bronchial epithelial cells in asthmatics (177) and TLR4-mediated signaling is involved in the genesis of airway inflammation in obstructive airway diseases (137). However, enhanced expression of these receptors does not appear to confer an advantage to replication of RSV in adult cells because there is no detectable difference in viral load between healthy and asthmatic epithelium.

A Causal Link between RSV Infection and Asthma

In addition to lower airway obstruction, infant hospitalizations due to RSV are associated with chronic wheeze and asthma in children (178–181). A recent prospective and interventional clinical trial that compared placebo- to palivizumab-treated patients suggested that RSV infection is causative of long-term wheeze in preterm infants (181). The complexity of the immune response and its change during development provide a link between wheeze, asthma, and RSV infection. This association may involve a key cytokine called IL-33. There is a large body of support for IL-13 as an independent and "pivotal" cytokine in the genesis of asthma (reviewed in reference 182). IL-33 may be involved in asthma genesis (183) and not just asthma exacerbation as with other models of infection, such as rhinovirus-induced asthma exacerbations that have been reported (41) (Fig. 4). IL-33 is a cytokine in the IL-1 family that stimulates production of type 2 cytokines from Th2 cells, basophils, eosinophils, mast cells, and nuocytes (42). The most notable type 2 cytokines stimulated by IL-33 are IL-4 (184) and IL-5 and IL-13 (185, 186), which are associated with the severity of asthma (187; reviewed in reference 188). RSV induced the expression of IL-33 in the lungs of neonates but not in the lungs of adult mice. IL-33 levels in the airways of mice during primary RSV infection as

neonates determined the severity of asthma in the mice as adults (183). The increase in IL-33 production during RSV infection resulted in an increase in group 2 innate lymphoid cells and their production of IL-13 (189). IL-33 binds the IL-33 receptor ST2 on nuocytes, triggering the production of IL-4, IL-5, and IL-13 (189), cytokines that are implicated in asthma genesis. Although an IL-33 producer cell has not been implicated in the pathogenesis of asthma, it should be noted that of all the different types of epithelial cells, bronchial epithelial cells express the most IL-33 in humans (42).

RSV and Bacterial Colonization

Airways that have been damaged by viral infections are susceptible to secondary bacterial infections (190) (Fig. 4). In a series of studies examining patients admitted to hospitals with RSV infections, between 17.5 and 44% of patients also tested positive for a lower respiratory tract bacterial coinfection, with *Streptococcus pneumoniae* (Gram positive) and *Haemophilus influenzae* (Gram negative) being the most common bacterial isolates (191–195). Compared to RSV infection alone, a bacterial coinfection combined with RSV infection correlates with more severe disease (64, 191, 192, 194). In addition to bacterial coinfection, the degree of nasopharyngeal colonization by Gram-negative or potentially pathogenic bacteria correlates with inflammatory cytokine levels and disease severity during RSV infection (191, 196, 197). Despite the connections between bacterial colonization and RSV severity, evidence does not support administering antibiotics to patients hospitalized with RSV infection in the absence of a specific concurrent bacterial infection such as septicemia, bacterial pneumonia, or otitis media (198, 199).

Instead, preexisting and sustained colonization by potentially pathogenic bacteria may make the host more susceptible to subsequent RSV infection. It has been shown *in vitro* or *in vivo* (using the cotton rat model) that treatment with *S. pneumoniae* prior to RSV challenge results in enhanced RSV replication (200). The increased RSV replication is also observed *in vitro* after pretreatment with heat-inactivated *H. influenzae* (201). Additionally, lipopolysaccharide (LPS; Gram-negative bacterial cell wall component) exposure results in a Th2 immune polarization and increased severity during subsequent RSV challenge (197). With further study, nasopharyngeal bacterial load and constituents may be utilized as an identifiable factor to detect infants at risk for severe RSV. In support of this idea, treatment with a multivalent pneumococcal vaccine is capable of reducing the subsequent incidence of RSV-associated pneumonia, indicating that at least a portion of viral pneumonia cases are due to either precolonization or concurrent infection by *S. pneumoniae* (202). However, the mechanisms of these interactions are still being elucidated. Furthermore, RSV infection increases bacterial binding to the cell surface and has been linked to decreased cilium movement (ciliary dyskinesia) (203–207), which may hinder bacterial clearance from the lower respiratory tract. RSV infection increases bacterial binding by causing infected cells to upregulate cell surface expression of a number of bacterial receptors, including intercellular adhesion molecule 1 (ICAM-1), platelet-activating factor receptor (PAF-r), and carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) (204, 206), and bacterial binding to RSV-G glycoprotein expressed on the surface of infected cells or on viral particles (206–209). The increased bacterial binding and ciliary dyskinesia together have been postulated as a mechanism for longer bacterial persistence in the lower respiratory tract during RSV infection and may explain the high likelihood of bacterial coinfection. In summary, a positive feedback loop may exist wherein RSV infection enhances bacterial colonization of the lower respiratory tract, which in turn increases RSV replication.

RSV SEROLOGY

Serologic Testing

Serological testing is not widely used for the diagnosis of RSV. Acute- and convalescent-phase serology paired with PCR increases the diagnostic yield of RSV, in patients 5 years of age and older, from 8.3% with PCR alone to 11.3% with acute- and

convalescent-phase serology (210). Enzyme immunoassays (EIAs) against envelope glycoproteins have a sensitivity greater than HEp-2 tube culture. However, the time required for a serological response assay (211) and comparison between paired and convalescent-phase serum samples has not been useful for guidance of patient care (http://www.cdc.gov/rsv/clinical/). Antibodies to glycoproteins F and G are thought to be neutralizing *in vitro* and in animal models and are used as correlates of vaccine/immune protection (212, 213). Neutralization assays conducted on participant postvaccination sera also play a significant role in vaccine clinical trials (214). RSV vaccine approaches usually target the development of protective antibodies, so viral neutralization is an accepted method to quantify the protective ability of antibodies in human sera (215).

Neutralizing Antibody Correlates of Protection against RSV Type A and Type B

The dominant neutralization epitopes on RSV exist on the RSV-F glycoprotein. Neutralizing antibodies have been determined using microneutralization assays but could not be predicted against the F protein when enzyme-linked immunosorbent assays (ELISAs) were used (216). A dominant neutralization epitope on the RSV particle, called site Ø, exists only on the metastable prefusion RSV-F protein and not the postfusion F protein (Fig. 2) (217). There are a number of other immunodominant sites on the RSV-F protein that can neutralize RSV infectivity, the most notable of which are sites I, II, and IV (218). Site II is the target site of palivizumab, the only licensed prophylaxis against RSV (219) (Fig. 2A). The structure and location of these sites on the RSV-F protein have been elucidated (94, 100). Since the dominant neutralization site Ø exists only in the prefusion state of RSV-F, there is now focused attention on the use of RSV-F prefusion conformational protein analogues to direct the development of prefusion F-based RSV vaccines (220). We discuss vaccine targets in more detail in Vaccines.

Protection from RSV has been described in other studies using complement-enhanced plaque reduction neutralization assays (221). Neutralizing antibodies to F and G proteins were identified as being short-term correlates of RSV protection in adults (222). This is likely because RSV infection either evades or suppresses the development of mucosal IgA memory responses in adult humans (223). The mechanism of RSV evasion or suppression of memory B cells remains to be elucidated (reviewed in reference 84). Influenza virus infection, in stark contrast, induces strong IgA B cell memory that is correlated with protection (223–225). Habibi et al. showed that mucosal IgA memory B cell responses correlated most closely with protection from RSV infection and that there was significantly less correlation of circulating neutralizing antibodies with protection from RSV infection (223). Therefore, the nature of neutralizing antibody responses and evasion of B cell memory by RSV has very significant implications in the development of RSV vaccines. However, the efficacy of many RSV vaccines is still being reported in terms of circulating neutralizing antibody titers (226). Current knowledge therefore dictates that vaccine efficacy should be reported as mucosal neutralizing antibody titer because this would appear to be the most accurate endpoint measure of RSV neutralization (223).

EPIDEMIOLOGY AND CLIMATE CONSIDERATIONS

RSV Worldwide Prevalence and Seasonality

It is suspected that almost everyone has experienced an RSV infection by 2 years of age, and it is estimated that 2% of those infected with RSV in this age range require hospitalization (3). As such, RSV infection accounts for almost 25% of all hospitalizations due to lower respiratory tract infections in North America (227). Most infections occur in seasonal outbreaks worldwide, between October and May in the Northern Hemisphere, that tend to coincide with influenza seasons (228). However, RSV seasons also tend to follow the rainy seasons in the Northern and Southern Hemispheres, and so an outbreak of RSV does not always coincide with influenza seasons (229, 230).

In North America, RSV infections will typically appear in October or November, with a peak between January and February, and the last RSV infections occur in the spring

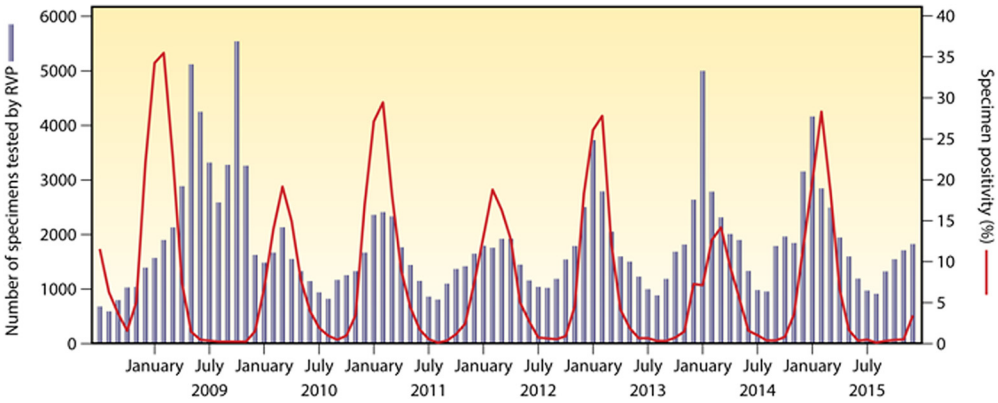

FIG 5 Seasonality and positivity rate of RSV in Alberta, Canada, 2008 to 2015. The graph indicates RSV test-positive specimens and overall respiratory virus test volumes by Luminex RVP classic assay. Data show specimens that were influenza virus negative and do not account for mixed infections by RSV and other pathogens. Peak periods occur in winter and early spring, with positivity for RSV ranging from 15 to 35% of all specimens tested by RVP.

(231). This is a pattern that we see commonly in northern Alberta, Canada, as well (Fig. 5), with changes in amplitude of numbers of specimens as well as specimen positivity on a year-to-year basis. In more tropical climates (Florida, USA, 2012 to 2013, for example), there tends to be an elongation of the season with an earlier start, since the first cases are reported by midsummer (231). There is evidence for distinct RSV seasons in tropical climates, which challenges the notion that the tropics experience a consistent level of RSV infections year-round (232). In general, RSV hospital admissions peak when yearly temperatures are at their lowest and precipitation is highest, which is likely due to indoor crowding that leads to higher transmission rates in temperate countries (3, 229). In most jurisdictions, RSV epidemiology does not differentiate RSV type A from RSV type B when reporting patterns of circulation, and so it is unclear if patterns of circulation differ between subtypes on a systematic basis.

Climate and Interactions between RSV and Other Viruses in the Population

In temperate climates, RSV infections occur in the winter months, starting in October and November in the Northern Hemisphere and typically peaking in January and February. The last infections of the season tend to occur between March and May (Fig. 5). Enterovirus infections peak in the summer in temperate regions, whereas influenza virus, RSV, and human metapneumovirus (hMPV) infections peak in the winter months (233, 234). This pattern of outbreaks is suggestive of a sociological and/or a meteorological effect on the timing of virus outbreaks. In northern Alberta, Canada, we see decreases in enterovirus infections to undetectable levels that coincide with a rise in the RSV and influenza season (Fig. 5). In further support of a meteorological influence on respiratory viruses, RSV and influenza seasons occur at discordant times of the year in Australia, coinciding more with the rainy and winter seasons, respectively (229). Recent epidemiological reports have suggested that there may be virological interactions such as competition (234) between influenza virus and RSV infections (233, 234). The early onset of an influenza season and the 2009 H1N1 influenza pandemic coincided with the delayed onset of RSV and hMPV epidemics (233, 234). It was suggested that epidemiological studies such as these support a hypothesis of viral competition and/or virological interaction (234). However, it is well known that a number of different combinations of respiratory virus coinfections occur; examples include RSV-influenza virus and RSV-hMPV coinfections (138). Furthermore, support for any mechanism of virological competition between influenza virus and RSV would require *in vivo* studies in an appropriate model or carefully controlled prospective clinical trials of influenza virus and RSV coinfections. There are also hypothesized interactions between RSV and other important bacterial respiratory pathogens that we discuss in "RSV and Bacterial Colonization" (207). Although it cannot be ruled out until

appropriate hypothesis-driven human trials and/or *in vivo* experiments are conducted, there is no way of discerning virological competition between RSV and influenza virus infections from epidemiological studies.

RSV Infections in Subtropical and Tropical Regions

There is evidence for distinct cyclic RSV seasons in equatorial and tropical countries (235). In South American equatorial countries, temperature and humidity were independently associated with the frequency of RSV infections (236, 237). However, RSV outbreaks were only loosely or not at all associated with temperature, humidity, and precipitation when tropical sites worldwide were compared in a given RSV season. Furthermore, in a given year, the timing of RSV outbreaks varied significantly between tropical sites, suggesting that even though there are outbreaks of RSV in tropical regions, it is difficult to predict RSV seasonality in these zones (235). In summary, as in temperate regions, the optimal conditions for transmission of RSV infection in tropical and subtropical countries tend to be when temperature and humidity are at their lowest, but there are other factors that influence RSV outbreaks in these climates (238, 239).

RSV Type A versus Type B

RSV type A (RSVA) and type B (RSVB) are the two subtypes of RSV that were first typed based on antigenic reactivity to monoclonal antibodies (240, 241). RSVA is, generally speaking, more prevalent than RSVB (242, 243), although a higher prevalence of RSVB than of RSVA is reported periodically (243–245). The reasons for the discrepancies in prevalence between RSVA and RSVB are not clear, although periodic models of alternating type A and type B epidemics have been proposed (246). Immunological diversion by an RSV type during one season may render the population more susceptible to the other type in subsequent seasons (246). Whatever the cause of alternating epidemics of RSV types, it is generally well accepted that RSVA viral loads tend to be higher than RSVB viral loads by about a logarithm (247). The higher viral loads of RSVA in the nasopharynx may enable faster transmission between individuals because more virus is shed for digital inoculation via the eyes and nose (248, 249).

The rates of spread and the virulence levels of RSV types have been compared by plaque assay in tissue culture (247). A unique report by Kim et al. compared the morphologies of RSV plaques derived from pediatric patient isolates (247). These were cultured viral isolates, so the plaque morphologies that were observed were the product of intrinsic features of the virus's own genomic constitution. Kim et al. reported larger plaque sizes caused by RSVA isolates than by RSVB (247), and there was a distinct difference in plaque morphology between RSVA and RSVB isolates (247). However, the authors reported no significant correlation between clinical severity and plaque size or morphology of type A and type B viruses using a classic plaque-clearing assay (247). Some RSV isolates can be poor at causing plaque clearings in cultured cell monolayers, so it would be interesting to study plaque size and morphology using an antibody detection assay of RSV plaques. In summary, comparing plaque size and morphology of RSV isolates is a good start toward understanding the differences in virulence between RSVA and RSVB.

Circulating RSV Clades and Strains

Genome sequencing has refined our knowledge of the clades and strains of RSVA and RSVB that circulate worldwide (15, 233, 250). There have been a few good reports on whole-genome sequences of RSVA and RSVB that describe the transmission of RSV genotypes derived from these two groups (233, 243, 250–252). The current working model of RSV transmission is that yearly outbreaks of RSV are the result of variants that grow out of locally evolved clades, not necessarily viruses that have been introduced from distant locations (233).

Initially, 5 RSVA clades and 4 RSVB clades were identified, named GA1 to GA5 and GB1 to GB4, respectively (244). This list of clades has since grown to 16 RSVA clades and

22 RSVB clades (253). A recent global survey identified GA1, GA2, GA5, and GA7 as the current major circulating clades of RSVA worldwide. GA7 is a major circulating clade of RSVA that is found only in the United States (251). The BA clade of RSVB predominates worldwide (251). In a major metropolitan center in Alberta, Canada, we are also detecting locally developed clades of RSV that reflect the clades reported by Bose et al. (251).

Our own surveillance and whole-genome sequencing (WGS) have shown a strong RSVB presence in addition to RSVA strains in northern Alberta, Canada (unpublished observations). In northern Alberta, we have found that RSVA is represented strongly by the GN435 strain and RSVB is mainly represented by the BA2013 strain, which is consistent with a worldwide RSV surveillance report that was published previously (251). Some studies have even shown a higher prevalence of RSVB infections in past seasons (243), similar to what we observe in northern Alberta, Canada.

Off-Season Virus Reservoirs

If yearly epidemics of RSV are derived locally, this begs the question of who is the RSV reservoir in the off-season when there are no detectable cases of RSV in the clinic. There must be underlying replication of RSV in pediatric or adult hosts during the summer. However, we were unable to find prospective sampling studies that sampled otherwise healthy individuals to identify an RSV reservoir. Therefore, we can only postulate that virulence may follow enhanced transmission when the weather turns cold and relative indoor humidity is decreased, favoring stability and transmission of RSV (254). The innate antiviral immune response is also blunted in the host's nasal epithelium in cold temperatures (255), possibly rendering a population of hosts more susceptible to contracting RSV infections and leading to more rapid spread of the virus, thus precipitating an RSV season. Enhanced surveillance of RSV reservoirs is needed if there is to be an efficacious vaccination program. It will be important to identify those reservoir viruses that are most virulent to help prospectively refine the next season's RSV vaccine.

It is interesting that RSVA and RSVB have remained two distinct subtypes despite the ability of RSV to mutate. Therefore, there may be a niche age range or demographic that each of these viruses has evolved to infect during active RSV seasons or reservoir-specific age ranges in the off-season. More research is still clearly needed in this area.

DIAGNOSTICS

Molecular diagnostic testing has formed the foundation of our knowledge regarding the frequency and prevalence of infection by respiratory syncytial viruses both worldwide (256) and in northern Alberta, Canada (Fig. 5). This knowledge then plays a critical role in aiding research studies and individual patient diagnosis and treatment. The traditional approaches to respiratory virus disease management have often focused on syndromic management instead of laboratory-focused diagnosis. If testing was done, it was done using a variety of approaches, including antigen detection (e.g., point-of-care [POC] testing or direct fluorescent antibody [DFA] technique), virus propagation in cell culture, and then viral characterization. The first patient testing point was often a first-generation POC test.

First-Generation Point-of-Care Tests

First-generation point-of-care (POC) tests can be classified as direct antigen detection assays that required the user to make a visual discrimination of antigen detection and were generally based on chromogenic immunoassay technology. These antigen detection systems suffered from poor test characteristics, including hampered sensitivities and specificity compared to multiple standard methods (Table 1). A recent survey indicated that in 2011, two-thirds of U.S. diagnoses of RSV were made by rapid antigen detection methods (R. Turner, B. Saunders, and L. Edelman, presented at the 28th Clinical Virology Symposium, Daytona Beach, FL, USA, 20 to 21 April 2012) (257). Rapid assays are generally available in three formats, immunochromatographic (ICR)

tests, enzyme immunoassays (EIAs), and optical immunoassays (OIAs), and most assays target the RSV-F surface glycoprotein (257) and nucleocapsid proteins of RSV (258).

First-Generation POC Test Characteristics in Pediatric Patients

In a pediatric population, a rapid chromatographic immunoassay (CIA) POC test was compared to reverse transcription-PCR (RT-PCR) for RSV (Directigen EZ RSV; Becton Dickinson, Sparks, MD). It had a sensitivity of 79.8% and a specificity of 89.5% (Table 1). Other first-generation POC assays have also had lower published levels of sensitivity compared to composite reference standards even when molecular reference methodologies were not used. Although good to excellent specificities for CIAs have been described in the literature, cross-reactivity with other viruses (e.g., hMPV) and high false-positive rates have been described compared to reference standards of direct fluorescent antibody and RT-PCR (259). Thus, it became evident that first-generation POC tests based on CIA technology needed significant improvement in sensitivity and specificity, and so they could not be used as a definitive diagnosis of RSV infection on their own (259, 260).

First-Generation POC Test Characteristics in Adult Patients

Historically, our lack of knowledge about RSV in adults may have been hampered by broad use of first-generation POC tests. Depending on the jurisdiction, these assays lacked regulatory clearance in adults (261), and the sensitivity of these assays for detection of RSV in adults is very poor (e.g., $<$20%) compared to a composite standard of cell culture, serology, or molecular detection methods (262). A recent review of laboratory testing trends for RSV in the United States indicated that in 2011, antigen detection was still the leading testing approach used in American hospital laboratories. For this period, molecular testing for the detection of RSV increased to greater than 20% of all tests reported in this period, with authors suggesting that this would have a beneficial impact on RSV surveillance data while indicating concern that systems primarily utilizing first-generation POC tests may have hampered surveillance systems (263).

Second-Generation POC Tests

Recent antigen detection tests utilize specialized detection approaches to improve test characteristics. As shown in Table 1, these tests have improved sensitivities and specificities compared to molecular assays. For example, the Sofia RSV assay has a sensitivity and specificity of 78.6% and 93.9%, respectively, compared to molecular assays (264) (Table 1). This fluorescence immunoassay uses a virus disruption step prior to detection of viral nucleoproteins and is designed to detect RSV from nasopharyngeal swabs and aspirates from pediatric patients less than 19 years of age but not from adults or immunocompromised patients. As shown in Table 1, these improvements in POC test characteristics are not kit dependent and similar improved sensitivities and specificities are seen with the BD Veritor RSV POC assay, which has a sensitivity of 81.6% and a specificity of 99.1% (265). This chromatographic assay, which detects the RSV-F glycoprotein, is intended for use on nasopharyngeal washes, aspirates, and swabs from patients under 20 years of age. With both described second-generation POC tests, negative specimens should be verified with another method (e.g., viral culture or an FDA-approved molecular test) according to the manufacturer's instructions. It should be noted that both described systems have reduced sensitivities for RSVA and -B compared to real-time PCR assays (266). Furthermore, compared to culture and some molecular tests, these second-generation POC assays suffer from the inability to distinguish RSVA from RSVB.

Clinical Laboratory versus POC Testing

POC tests are often more rapid and convenient than tests that require sample submission to a clinical laboratory. With the centralization of clinical microbiology laboratory services in many jurisdictions, the submission of a patient sample to a

TABLE 1 Diagnostic methods for detection of RSV in patient populations[a]

Test type	Assay	Study test population age (yr)	% sensitivity	% specificity	Comparison method	Reference
First-generation POC	Binax Now	All ages, all specimens (0 to >89)	70.8	97.8	Composite standard: cell culture and DFA	359
	Binax Now	<22	72.0	97.6	Composite standard: cell culture and DFA	359
	Binax Now	<5	72.4	97.6	Composite standard: cell culture and DFA	359
	Binax Now	Pediatric	90	100	Composite standard: cell culture and/or PCR	360
	Binax Now	Not defined	81.7	98.7	Composite reference	361
	BD Directigen EZ RSV	Pediatric	90	94	Composite standard: cell culture and/or PCR	360
	BD Directigen EZ RSV	Pediatric	79.8	89.5	Laboratory-developed RT-PCR	259
Second-generation POC	3M rapid detection RSV	All ages, all specimens (0 to >89)	86.3	95.8	Composite standard: cell culture and DFA	359
	3M rapid detection RSV	<22	87.2	95.6	Composite standard: cell culture and DFA	359
	3M rapid detection RSV	<5	87.9	95.8	Composite standard: cell culture and DFA	359
	BD Veritor system	<6	81.6	99.1	Prodesse ProFlu$^+$ RT-PCR	265
	BD Veritor system	<6	79.1	96.8	Laboratory-developed RT-PCR	362
	RSV K-SeT antigen test	<6	79.1	95.8	Laboratory-developed RT-PCR	362
	Sofia	<18	87.7	94.7	Traditional cell culture	363
DFA on primary specimen	SimulFluor	0–17	93.5	99.6	Composite reference	364
	Cytospin- DFA	Mostly adult hospitalized patients	73.9	99.8	Laboratory-developed RT-PCR	271
	Bartels; Trinity Biotech	Pediatric	94.1	96.8	Laboratory-developed RT-PCR	259
Culture	R-Mix Too followed by IF	Not defined	63.2	ND	Composite reference	294
	R-Mix followed by IF	0–17	86.5	100	Composite reference	364
	WI38, RMK, and D^3 Ultra DFA respiratory virus screening and ID kit	Not defined	56.9	100.0	Composite reference	361
Rapid molecular test	Cepheid Xpert Flu/RSV	Not defined	97.9	100	Laboratory-developed RT-PCR	274
	Cepheid Xpert Flu/RSV	Not defined	90.6	99.4	Laboratory-developed RT-PCR	365
Multiplex molecular	AdvanSure	Not defined	96.8	100	Composite reference	294
	Seeplex RV15 ACE	Not defined	94.7	100	Composite reference	294
	ResPlex II Panel v2.0	0–17	84.0	100	Composite reference	364
	Seeplex RV15	0–17	100	97.7	Composite reference	364
	xTAG RVP	0–17	88.2	100	Composite reference	364
	xTAG RVP Fast	0–17	91.7	100	Composite reference	364
	xTAG RVP Fast	0–84	94.7	99.2	Laboratory-developed real-time RT-PCR	366
	Simplexa FluA/B and RSV	Mostly adult hospitalized patients	91.3	98.9	Laboratory-developed RT-PCR	271

[a]Abbreviations: ND, not defined in study; IF, immunofluorescent staining; ID, identification.

centralized clinical laboratory can require hours to days before a definitive answer to the etiology of a patient's symptoms is returned to the health care provider (267). One dilemma is that unless POC test utilization is carefully planned and the data are logged in health care systems, there may be a loss of data flowing into surveillance programs. On the other hand, the use of POC test data (especially from tests with improved test characteristics) in well-planned surveillance systems may effectively alert clinicians and public health authorities of impending and current outbreaks. Surveillance data derived from clinical laboratories are also frequently used by health care delivery organizations and public health to appropriately allocate health care funds in hospitals and the community for the purchase of therapeutics, supplies, and diagnostics that support appropriate treatment(s) (268).

Direct Fluorescent Antibody Testing

Direct fluorescent antibody (DFA) testing requires a swab that allows for an appropriate number of epithelial cells to be collected and is largely applicable to appropriately collected nasopharyngeal specimens. Specimens that lack enough cells or originate from other sites in the respiratory tract are not appropriate for this type of testing. This is suggestive that historic surveillance data that relied heavily on DFA testing may have been underestimating the true impact of RSVA and RSVB on influenza-like illness rates in pediatric and adult populations, and rates will change as surveillance systems shift between different detection approaches (263). However, depending on workflow and resources within the laboratory, DFA testing as an adjunct to molecular test methods may provide an option for RSV testing in high-risk patients such as hematopoietic stem cell transplant patients (269). Prior to the broader utilization of easier-to-use molecular diagnostic assays, DFA testing historically provided a more rapid response than lab-developed and batched molecular assays for RSV (270). As shown in Table 1, the sensitivity of DFA testing can be excellent in pediatric patient populations. However, as seen with first-generation POC testing, the sensitivity of DFA testing is significantly decreased in adult patient populations, especially compared to commercial nucleic acid amplification tests (Table 1) (271). It should be noted that not all molecular assays are equivalent for the detection of respiratory viruses, and our own experience has occasionally identified instances where DFA testing was able to identify RSV while molecular test results were variable (unpublished observations). Other groups have identified that in pediatric populations, compared to nucleic acid amplification testing, DFA test sensitivities are probably highest in the first 3 days of infection (272).

Crossing the Divide between Third-Generation POC Tests and Low-Complexity Lab-Based Molecular Tests

There is no FDA-approved molecular POC test for RSV currently available for diagnostic use in the United States. However, with the recent clearance of an influenza A/B virus POC molecular test in January 2015 (273), it should be expected that a similar assay for RSV will be developed in the near future. Once available, these types of tests would fall within the definition of third-generation POC tests: molecular assays that have the ability to be performed in low-complexity test environments close to patients. They include integrated nucleic acid extraction, amplification, and detection systems. Although they are generally unable to handle high workflows, these tests have excellent sensitivities and specificities compared to molecular tests run in high-complexity laboratories. Several assays are available internationally, which might be available in North America in the near future; common themes include integrated extraction, detection, and interpretation with limited hands-on time. Currently, the types of assays closest to third-generation/molecular POC assays would be the assays identified as moderate-complexity assays by the FDA (http://www.cdc.gov/flu/pdf/professionals/diagnosis/table1-molecular-assays.pdf). For example, from our experience, the Cepheid Xpert Flu/RSV XC assay is relatively easy to operate and has published sensitivity and specificity of 97.9% and 100%, respectively, for RSV compared to a laboratory-based

molecular method for the detection of RSV (274) (Table 1). Other similar technologies such as Enigma, a laboratory-based RSV assay which detects the RSV-F gene, can be carried out in a single cartridge and provide a sample-to-answer result within 95 min (Enigma Diagnostics, San Diego, CA). Recent publications indicated high sensitivity, specificity, and positive and negative agreement with laboratory methods for the detection of RSV (275, 276).

Commercial Molecular Methods for Detection of RSV

Commercial high-complexity assays with FDA approval are identified in a table by the FDA (http://www.cdc.gov/flu/pdf/professionals/diagnosis/table1-molecular-assays.pdf). Detection of RSV in these assays may be coupled to a relatively small number of targets (e.g., influenza A/B virus) or may occur in extensively multiplexed panels (e.g., eSensor respiratory viral panel [RVP] and Luminex RVP panels). While some assays such as the Verigene respiratory virus nucleic acid test allow for discrimination between RSVA and RSVB, other assays do not distinguish between these RSV types (e.g., Prodesse ProFlu). Issues with the use of these types of commercial assays include the requirement for substantial training, quality systems, and infrastructure to maintain and run these assays. Many of the tests' characteristics are similar between the commercial assays (277). The proprietary nature of these assays typically means that specific target locations or even target gene information is not made available to laboratories to assist with troubleshooting. However, these systems are convenient and thus beneficial in allowing for improved laboratory workflow, scalability/surge capacity, and, in many cases, linkages with laboratory information systems. Many of the commercial tests that have received FDA or Health Canada approval may have been approved for only nasopharyngeal swabs or aspirates. This means that specimens from other respiratory tract sites, or even nasopharyngeal washes, may be required to undergo extensive laboratory validation and verification.

Newer trends in commercial nucleic acid amplification tests for RSV involve single-step nucleic acid extraction and amplification in a single platform (278). Advertised sample detection in these assays is approximately 1 h and therefore significantly faster than multistep assays like RVPs that take upward of 8 h to produce results.

Detection of Multiple Respiratory Pathogens in High-Complexity Assays

Multipathogen panels like the eSensor RVP by Luminex will commonly detect the presence of multiple virus infections in a given patient. A dominant virus paradigm was postulated in the work of Utokaparch et al., which suggests that when multiple viruses are present simultaneously, one virus infection tends to dominate over the others in terms of viral load (138).

Commercial versus Laboratory-Developed Nucleic Acid Amplification Test Systems

A variety of publications have identified laboratory-developed nucleic acid amplification test systems for the detection of RSVA and RSVB in clinical specimens. The approaches may utilize PCR-based technologies (279) or other approaches such as reverse transcription–loop-mediated isothermal amplification (RT-LAMP) (280). Gene targets of laboratory-developed molecular assays may vary but can include the M gene (280) and the N gene in RSVA and -B (279). Benefits to laboratory-developed assays include flexibility to modify assays when targets are under evolutionary pressure to change, as well as a perceived initial low cost to carry out testing. However, laboratory-developed test systems generally require the laboratory to carry out a higher level of complexity of testing and troubleshooting than with commercial products. Laboratory-developed assays also require significant validation and verification compared to FDA- or Health Canada-approved assays, and this is often not an insignificant investment (281). If not established properly (e.g., automation and data flow to laboratory-information systems), these systems may also not allow for high-throughput scale-up compared to commercial systems (282).

RSV Culture as a Nonbiased Option for Reference Laboratories

Culture approaches, including shell vial culture approaches, have improved test characteristics compared to the second-generation POC assays (e.g., BD Veritor) for the diagnosis of RSV in pediatric patients (283). Historically, there have been a variety of culture approaches used in RSV diagnostics, including tube and shell vials followed by a direct fluorescent antibody detection or enzyme immunoassay detection approach. A variety of cell lines have been used in clinical laboratories, including human embryonic kidney and HEp-2 (284) tube cultures and shell vials (285), A549 conventional and shell vial cultures (286), and rhesus monkey kidney (RhMK) and human foreskin fibroblast conventional cultures and shell vials (287, 288). Commercially available products such as R-mix shell vials, a combination of mink lung cells (Mv1Lu) and human adenocarcinoma A549 cells, have been used to support and allow for the detection of RSV when paired with an antibody detection method (283, 289). Occasionally but not routinely, RSV propagation has been described in H358 (290) and human adenocarcinoma MRC-5 (291–293) cell culture tubes. However, culture approaches lack sensitivity, often quite significantly, compared to nucleic acid amplification assays for the diagnosis of RSV infections (Table 1) (294, 295). This was also notable when nucleic acid amplification test methods were compared to culture for lower respiratory tract specimens (296). In our own experience, culture of RSV from previously frozen nasopharyngeal samples from patients with a relatively high quantitative reverse transcription-PCR (qRT-PCR) viral load has proven difficult. If the sample is frozen after collection, then culture is almost impossible. We have interpreted these aspects of RSV culture as representative of the unstable and labile nature of the viral particles themselves.

Given the relatively inconsistent track record of RSV culture, there has been a notable decrease in the use of culture-based detection techniques in U.S. hospital-associated laboratories, as the use of molecular tests has increased, especially from the period 2007 to 2011 (263). However, there have been descriptions of multiplexed PCR assays with poor test characteristics compared to other PCR methods or even culture (297). Until nonbiased detection systems become more widespread and reliable, culture- and antigen detection-based assays play an important backup role especially in cases where single nucleotide polymorphisms lead to false-negative PCR results (298).

Other roles for culture may include propagation of virus for further genetic characterization, a trend that may decrease with whole-genome sequencing, and analysis of antiviral compounds (299, 300) and virucidal agents (301).

Whole-Genome Sequencing and Next-Generation Sequencing

Next-generation sequencing (NGS) or whole-genome sequencing (WGS) has not yet made its way to the clinical setting as a front-end diagnostic tool. Part of the problem is that RSV RNA genome sequences or the RNA/DNA genomic sequences of any other pathogen may not be the dominant target and may in fact be scarce within a clinical specimen. There are still no standard approaches to viral pathogen diagnosis (302). For non-RSV viral respiratory targets, NGS has had variable sensitivities compared to target-specific RT-PCR assays (303). Another NGS diagnostic approach is to use preferential amplification of pathogenic sequences (PATHseq) to identify nonhuman sequences (304). There are now multiple examples of NGS being used to diagnose viral infections in human patients in a nonbiased manner (305–307). A more routine role of NGS is thought to be possible with computing technology and decreases in cost (308). In the meantime, NGS will play an increasing role in identification of RSV and characterization of strain diversity in special cases (252). The public health role will include identification of evolutionary diversity and potential patterns of transmission while using primary clinical specimens (250). Also, apart from diagnostic roles, NGS of RSV has helped identify mutation rates for RSVA and -B as well as global circulation patterns of RSVA and -B clades (250). SNP analysis of RSV is indicating a significant number of SNPs in RSV-G and -F genes, while the P gene of RSV is more conserved (252).

Standardization of Detection Methods for Surveillance Purposes

Standardization of RSV detection methods will be required when an RSV vaccine is approved for broad dissemination. We have learned that RSV, consistent with being an RNA virus, will likely mutate amid selection pressure to evade an approved vaccine. A consensus on ideal RSV detection and surveillance technologies is clearly needed, preferably before a vaccine is approved for community administration. This will likely include a breadth of factors, including but not limited to specificity, sensitivity, processing time, complexity, and cost.

VACCINES

Introduction

There are a number of RSV vaccines in various stages of clinical trials as of the publication of this article. There are a number of excellent reviews that describe the current state of RSV vaccine development, such as reference 213, so we will discuss a few pertinent highlights of RSV vaccine development as opposed to providing an exhaustive review.

Lessons from Vaccine Strategies That Have Undergone Clinical Trials and Failed

A formalin-inactivated RSV vaccine was developed and underwent clinical trials in infants in the 1960s (10; reviewed in reference 309). The vaccine preparation in question enhanced RSV disease and ended with the deaths of two of the vaccinees (11). The reason for the enhanced respiratory disease was due, in part, to formalin-mediated destruction of neutralizing epitopes in the vaccine preparation (310, 311). The vaccine was immunogenic but induced poor antibody affinity maturation and poor antibody avidity for virus epitopes (12). Delgado et al. (12) argued that it was not necessarily formalin destruction of viral epitopes but insufficient TLR activation of B cells by the vaccine that led to enhanced disease in the 1966 trial (10, 11). They demonstrated that formalin- or UV-inactivated RSV vaccines became protective upon addition of TLR agonists poly(I·C) and lipopolysaccharide (LPS) to the vaccine preparations (12). Their hypothesis was supported by observation of deficient responses to a live RSV vaccine in mice deficient in the MyD88-TLR signaling pathway (12). In summary, the antibodies that were induced by the formalin-inactivated vaccine were of low avidity and low affinity and ultimately pathogenic due to poor TLR activation of B cells (12). However, these findings do not necessarily preclude a destruction-of-epitopes hypothesis but elaborate more mechanistically on epitope destruction. They elaborate on the failure to induce appropriate TLR activation in this vaccine preparation. Whatever the mechanism, these studies underline the need for RSV vaccines to be developed around rational subunit approaches that induce neutralizing antibodies to RSV in the airway mucosae. Some promising examples of strategies currently under development are stabilized prefusion RSV-F proteins and other subunit vaccines that preserve key neutralizing epitopes on the RSV-F glycoprotein (217, 226).

Subunit and Live-Attenuated Vaccines

Subunit RSV vaccines have demonstrated induction of stable B cell memory in mice (312). Those that underwent clinical trials between 1994 and 2001 were safe in healthy infants (313, 314), adults (313, 315–317), and individuals with preexisting pulmonary disease (318, 319). One of the preparations that underwent clinical trials was even a mixed subunit/attenuated RSV vaccine (316). These vaccines all elicited circulating humoral IgG titers that were neutralizing against RSV infection *in vitro*. In one study, it was noted that cell-mediated responses were elicited in children by a purified RSV-F protein vaccine but that this did not confer protection from RSV infection (314). One of the major problems that was identified in developing a live-attenuated vaccine was that a delicate balance between virulence of the vaccine strain and immunogenicity had to be struck to ensure a safe yet immunogenic vaccine. To date, none of those preparations mentioned above has proceeded to market as a protective RSV vaccine.

Prefusion RSV-F versus Postfusion RSV-F Vaccines

The principal neutralizing determinant on the RSV particle is the RSV-F glycoprotein (320). The RSV-F protein mediates the fusion reaction that causes mixing of the virus and host cell envelopes, leading to delivery of the virus capsid core contents into the cell (Fig. 2). The structures of pre- and postfusion RSV-F were recently elucidated (94, 100, 321). The RSV-F protein undergoes a number of conformational changes during the fusion reaction that bring the virus and host envelopes into close apposition. Disrupting the activity of this protein therefore disrupts viral entry and protects the host against infection.

The failure of previous RSV-F subunit vaccines led to rethinking of vaccine strategies and to the RSV-F prefusion conformation specifically. The conformation of the RSV-F protein prior to fusion is termed prefusion F, and it exists as a trimer on the surface of the RSV virion. The latest and most promising experimental vaccination strategies are based on a conformational analogue of prefusion F, as this form elicits the most potent neutralizing antibodies against RSV, which are reportedly 10- to 100-fold more potent than palivizumab (94).

Vaccines in Clinical Trials

There are currently several recombinant RSV subunit vaccines in clinical trials. Here, we mention one of the vaccines that garnered the most attention recently. In February 2016, Novavax (226) developed a vaccine that completed a phase II clinical trial. The Novavax vaccine is an RSV-F nanoparticle (RSV-F protein-liposomal) preparation. In September 2015, Novavax reported that their RSV-F subunit vaccine elicited expression of circulating neutralizing antibodies against RSV. Unpublished preclinical experiments that were made available as conference proceedings (322) showed that the Novavax vaccine elicited transplacental transfer of maternal antibodies in baboons but failed to confer significant protection to the infants by vaccination of the mother. This same vaccination strategy was used in their clinical trial and was the basis of the recent Novavax vaccine announcement (226). Though the Novavax baboon study was apparently underpowered, similar levels of protection were observed using the same vaccination strategy in lambs born to vaccinated ewes (323). In this study, there was a mere unprotective 3-fold drop in RSV titer in the vaccinated group compared to the placebo group (323). Therefore, the next round of efficacy trials of the Novavax vaccine is being watched with cautious optimism.

The efficacy of the Novavax vaccine was reported as a function of circulating neutralizing antibodies (226). RSV infection alone, without vaccine stimulation, will trigger production of robust neutralizing antibodies in the circulation and strong cytotoxic T lymphocyte (CTL) memory (reviewed in reference 84). However, it has not been reported whether the Novavax vaccine elicits mucosal neutralizing IgA antibodies that better correlate with protection (223). Thorough investigation of the IgA response is important since, as we mentioned above in the review, RSV infection evades IgA B cell memory through unknown mechanisms (223, 312, 324) and RSV is thus able to reinfect the host throughout his or her lifetime (1). So far, there is no indication that any of the vaccines currently in clinical trials elicit mucosal anti-RSV IgA neutralizing antibodies or long-term IgA B cell memory, two requirements for an RSV vaccine to confer a significant level of protection. In summary, it is too soon to tell whether the RSV vaccines that are in clinical trials will confer protection against RSV infection.

ANTIVIRAL THERAPEUTIC STRATEGIES

If an RSV vaccine is eventually licensed, there is a strong likelihood that there will remain a need for RSV anti-infective medications. As we discussed in "Circulating RSV Clades and Strains," RSV will most likely undergo vaccine escape mutation amid any active RSV vaccination program. In this scenario, active management of RSV infection in the very young, elderly, and immunocompromised will still be necessary. There are currently only two antivirals for RSV available, palivizumab for prevention and ribavirin for treatment.

Ribavirin as Treatment for Active RSV Infection

Currently, the only licensed drug for treating existing RSV infection is aerosolized ribavirin treatment of patients at highest risk from RSV infection. Numerous blinded trials of RSV-infected patients have demonstrated faster RSV clearance, decreased viral shedding, and shorter hospitalization stays with the use of ribavirin to treat RSV infection (325–327).

The hazard/benefit ratio of ribavirin to health care workers and patients has been questioned, however (328–331). The teratogenic effects of ribavirin in laboratory models (330, 331) and its cardiovascular contraindications at therapeutic doses mean that a cumbersome scavenging ventilation system is required for every ribavirin aerosol tent that is used to treat an RSV-infected patient (332, 333). In summary, safer drugs at lower therapeutic doses are needed to ensure that the next anti-RSV drugs see widespread use to prevent and treat RSV infection.

Intravenous Immunoglobulin and Monoclonal Antibodies for Treatment of RSV Infection

RespiGam was an RSV intravenous immunoglobulin (IVIG) licensed for prevention of RSV infection in premature infants and immunodeficient individuals (334, 335). It has since been withdrawn from the market after the introduction of monoclonal palivizumab (Synagis; discussed below). The IVIG in RespiGam was collected from the plasma of donors who had naturally high circulating levels of RSV-neutralizing antibodies. Naturally, pooled IVIG preparations also contained neutralizing antibodies to other viruses and bacteria, so in addition to preventing RSV infection, IVIG also prevented other respiratory virus infections and otitis media (334, 336).

The benefits of RSV hyperimmune IVIG have not been forgotten by the research community, as new preparations are being tested in subjects with primary immune deficiencies (337). A recent phase III trial identified the anti-RSV effects in addition to the broad antiviral and antibacterial properties of IVIG in patients with primary immunodeficiencies (337).

Licensed Monoclonal Antibody Antiviral Strategies for Prevention of RSV Infection

The biologic palivizumab (MedImmune, USA) is the only FDA-licensed drug that specifically targets RSV infection, and it has a benefit over RSV hyperimmune IVIG in that it can be delivered intramuscularly rather than intravenously. It is a humanized monoclonal antibody that is directed against the RSV-F fusion protein expressed on the surface of the RSV virion (338). Palivizumab and the closely related motavizumab bind to an epitope within amino acid positions 258 and 275 in the RSV-F protein. Palivizumab is licensed for RSV prophylaxis only in premature infants and those born with cardiopulmonary disorders (338). Currently, there is no efficacious treatment for active RSV infection.

Inhaled Nanobodies for Treatment of RSV Infection

Perhaps a next generation of monoclonal antibody treatment for respiratory disease is the inhaled nanobody (339). Nanobodies are derived from camelids (llamas, camels, and dromedaries) who make a heavy-chain-only antibody that differs in architecture from human antibodies. Most notably, when the variable domains, called V_{HH} fragments, of these camelid antibodies are expressed alone, they retain their specific antigen binding capacity. These therapeutic V_{HH} domains are called nanobodies.

A nanobody called ALX-0171 (340) has been developed against the RSV-F protein for the treatment of RSV (Table 2). This anti-RSV nanobody is an engineered trivalent preparation of V_{HH} domains derived from a monovalent V_{HH} domain that was selected for its ability to bind to antigenic site II on the RSV-F protein (Fig. 2) (340). There are promising preclinical data supporting the neutralization efficacy of ALX-0171, and it successfully completed a phase I clinical trial in healthy adults. The safety and pharmacokinetic data are available (341). ALX-0171 is currently being tested in infants less

TABLE 2 Experimental antiviral strategies

Antiviral target and mechanism	Antiviral agent	Model system(s)	Target or purpose	Reference
Virus particle inactivation	Silver nanoparticles	Cell culture	Virus particle inactivation	367
Viral replication/protein synthesis	Emodin	Cell culture	Biological synthesis inhibitor	368
	ALS-008176	Human challenge study	Nucleoside analogue	102
	Multiple small molecules	Biophysical approaches, crystallography	RNA-dependent polymerase complex	369
	Small-molecule inhibitor AZ-27	*In vitro* transcription run-on assay	RNA polymerase L	101
Virus cell binding	Glycosaminoglycan binding peptides	Cell culture	Block GAG-binding sites on the host cell	355
	Decoy liposomes	Cell culture	Target receptor pockets on viral binding proteins	356
Fusion and entry	GS-5806	Cell culture	Block virus entry, fusion inhibitor	370
	GS-5806	*In vitro* protein studies, clinical trial investigation	RSV-F glycoprotein	348, 371
	ABLX-0171 nanobody	Cell culture, clinical trials	RSV-F glycoprotein antigenic site II	340
Multiple mechanisms	DNA-conjugated gold nanoparticles	Cell culture	Entry, budding	357
	Flavonoids	Cell culture	Modulation of apoptosis and inflammation	358

than 23 months of age in a phase II trial that was started in early 2016 to test safety and efficacy (clinicaltrials.gov registration no. NCT02309320).

Mutations That Lead to Drug-Resistant RSV Serve as Lessons for RSV Vaccines

As we discussed above, RSV is an RNA virus, and so proofreading mechanisms to edit mutations in genome transcripts do not exist. As such, mutations that confer drug resistance to a virus variant will outgrow wild-type viruses amid antiviral selection pressure. Consistently, palivizumab-resistant RSV strains have been identified in experimental models (342, 343) and more recently in the clinic (344). Mutations at amino acid positions 272 and 275 of RSV-F were selected for *in vitro*, and mutations at these same sites were detected in RSV-breakthrough patients treated with palivizumab. Although in all cases the resistant virus variants had impaired fitness relative to the wild-type viruses and wild-type virus would outgrow drug-resistant variants upon removal of drug (344), these findings nevertheless provide an example of the capacity of RSV to evolve escape mutations to neutralizing antibodies. With this in mind, the administration of any RSV vaccine program will need a continuous surveillance arm established to identify vaccine escape mutants.

RSV Therapeutic Window Compared to That of Influenza Virus

New therapeutic options are required for the treatment of active RSV infection. We believe that RSV is a viable target for the development of antivirals, compared to viruses such as influenza virus, for example. Tamiflu is a neuraminidase inhibitor that has been marketed for the treatment of influenza (345), but the efficacy of Tamiflu has been questioned (346). This is due, primarily, to a brief therapeutic window of opportunity whereby Tamiflu must be administered prior to the peak of influenza viral load which is within 48 h of influenza virus infection (160, 223). Such a short window makes influenza difficult to treat because the onset of symptoms follows initial replication, leaving a mere few hours between transmission and therapeutic efficacy. With respect to the therapeutic window, RSV is an easier target than influenza virus because the peak of RSV viral load is much later, up to 8 days postinfection (139, 160, 161, 223). This leaves a number of days in the RSV therapeutic window to treat RSV, meaning,

theoretically, that RSV should be an easier infection to treat than influenza. In summary, alleviating the load of RSV in the airway with an antiviral will alleviate pathology caused by RSV infection.

Experimental Antiviral Therapeutic Strategies

A recent review provides an excellent summary of the challenges encountered in the development of RSV therapeutics (347). That particular review was written following two key scientific meetings on therapeutics, the Wellcome Trust-sponsored meeting of 2012 and the Global Virology Foundation meeting of 2013 (347). A key point of the review indicates that new diagnostic approaches will play a key role in the identification and subsequent enrollment of RSV-positive patients into clinical trials. Key groups that will benefit from this work will clearly be those described above in this review, both pediatric, elderly, and immunocompromised groups (347). Coordinated international groups are now emerging with the goal of reducing the burden of RSV disease globally (http://www.resvinet.org/about.html).

There have been several attempts to meet the need for RSV therapeutics, and there are a number of promising therapeutic strategies that have been developed for the treatment of RSV infection, summarized in Table 2. Some of these experimental therapeutic strategies have undergone clinical trials. For example, GS-5806 is an RSV fusion and entry inhibitor that successfully completed a phase I safety trial and a phase II RSV challenge trial in adults (348). It will be interesting to see whether GS-5806 can be used to treat RSV infection in pediatric patients, the elderly, and the immunocompromised. As shown in Table 2, however, the vast majority of these agents are still in the preliminary stages of research either in cell culture models of infection or in protein binding studies.

Nucleoside Analogues and Small-Molecule Inhibitors

From a historical perspective, nucleoside analogues that inhibit the function of the RSV polymerase are a rather obvious approach for the treatment of RSV infection. They were among the first FDA-approved treatments for HIV infection and are now the mainstay of HIV triple therapy (349). A more recently developed nucleoside analogue, marketed by Gilead Pharmaceuticals as Sovaldi, is now the basis of a cure for hepatitis C virus infection (350), with several more promising drugs and preparations in various stages of development. With regard to RSV therapeutic development, a robust *in vitro* RSV polymerase system (115, 117, 351) has been used to test nucleoside analogue libraries and other small-molecule inhibitors for their ability to inhibit the RSV polymerase complex. There is one nucleoside analogue named ALS-008176 (4′-chloromethyl-2′-deoxy-3′,5′-di-*O*-isobutyryl-2′-fluorocytidine) that was discovered using an RSV replicon readout system (352); it underwent successful clinical trials and was reported to inhibit RSV viral load by over 85% in human volunteers (102). Successful clinical trials are not a guarantee of safety, however. Hazards related to nucleoside analogue-based antiviral therapeutics have been discovered after initial safety trials were completed (353). A recent paper reported that nucleoside analogues with RSV antiviral activity were incorporated by mitochondrial RNA and DNA polymerases (354). In summary, efficacy and safety considerations of one successful hit are not sufficient to treat the spectrum of patients who are susceptible to RSV, so there is still a large unmet need for the development of more RSV antivirals.

Many different RSV preclinical therapeutic approaches are being developed (Table 2). Several agents such as glycosaminoglycan binding peptides (355) or decoy liposomes (356) interrupt the binding of the virus to receptors on the host cell membrane. Of the more preliminary agents under investigation, the most general mechanism of inhibitor activity is inactivation of the virus particle through interaction with an agent such as gold nanoparticles (357). Multipronged approaches are the combined inhibition of viral attachment, entry, and budding (e.g., DNA-conjugated gold nanoparticles). There are also host-directed therapeutic strategies being developed that modulate host

immune responses such as apoptosis and inflammation that favor viral clearance (e.g., flavonoids [358]) (summarized in Table 2).

CONCLUSIONS

There is an exceptional amount of knowledge on the basic mechanisms of RSV replication, transmission of RSV in the community, and clinical management of RSV disease. However, the progress made in RSV vaccines and therapeutics since the discovery of the virus in 1950 has been poor, particularly compared to other viruses like influenza virus, hepatitis C virus, and HIV. There is a tremendous amount that we know about RSV replication, and several steps in the replication cycle have been identified that can be exploited as antiviral therapeutic strategies. Our understanding of RSV replication is gradually leading to development of new therapeutics, and there will likely be new therapeutic developments trickling out as clinical trials continue. In summary, with all that we know about RSV and the prevalence, morbidity, and mortality caused by this virus, it is difficult to understand why there is such a disproportionate availability of prophylactic and therapeutic options to treat RSV disease.

The development of several rapid RSV detection tests over three generations has refined point-of-care testing. However, caution must be employed to ensure that the wealth of information on RSV infections in the community is not lost to unreported bedside testing. Carefully designed reporting programs that gather RSV diagnostic data from a wide variety of clinical sources are therefore needed.

A promising sign of things to come is the growing number of clinical vaccine trials that are being conducted. RSV has proven particularly adept at either suppressing or evading B cell memory, and so there is the question of whether a vaccine can be developed that will elicit lasting RSV-neutralizing antibodies in the mucosae. If these hurdles can be overcome, vaccination of prepartum mothers with a robust anti-RSV vaccine would likely confer significant protection through passive immunity in infants. Eventually, a number of vaccination options should become available for the protection of children and the elderly, who are also at significant risk from RSV infection.

Looking forward to efficacious RSV vaccines and therapeutics, one must consider active RSV surveillance. RSV infection surveillance through point-of-care and laboratory tests will be needed to monitor the effectiveness of new vaccines and therapeutics in the community. As discussed in this review, there is significant evidence to suggest that vaccine escape mutations will evolve that would require established and well-run RSV surveillance programs, on par with influenza vaccine monitoring, to refine existing vaccines and therapeutics.

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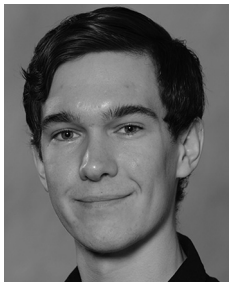

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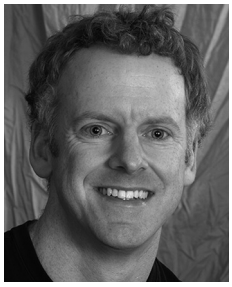

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